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## CIBA SEMINAR: RECENT ADVANCES IN HYPERSENSITIVITY

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### THE NATURE OF THE ANTIGEN AND THE BASIC CHARACTERISTICS OF THE IMMUNE RESPONSE

GEOFFREY EDSALL, M.D.,* WASHINGTON, D. C.

ONE can hardly cover the subject matter of this talk completely in twenty-five minutes. Much of it is certainly familiar to most of you. Also, much of it has been said before. Therefore, I shall undertake only to comment on a few of the points which I consider most significant—on some new findings which are of particular interest and some areas which deserve further exploration and in which the immunologist and the allergist can find much in common—on certain characteristics and properties of antigens, on the pattern of the immune response, on the effect of the size, number, and spacing of doses, on antigenic competition, and on a few host factors of interest, especially age.

Much of what is known about antigens has been known for a long time, and I have not been impressed by very much in the way of striking recent advances in knowledge of the nature of antigens. Furthermore, much of what should be said in this field was said beautifully by Boyd[1] in the JOURNAL OF ALLERGY eight years ago, and there have been several other good reviews or discussions in this general field since then. Therefore, what I will do in this part of the discussion is simply to comment on some of the well-known characteristics of antigens.

*Antigens.*—Antigens are usually defined by the second-year medical student as substances which produce antibodies. When the student is then asked to define antibodies, he is likely to say that they are substances produced in response to the stimulus of antigens. Sophomoric though this circular set of definitions may seem, it actually reflects to a considerable degree the limitations of our knowledge as to why antigens and antibodies are what they are.

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*Director, Division of Communicable Disease, Walter Reed Army Institute of Research.

We do know, of course in a general way, that antigens are invariably substances of large molecular weight and size—say from 10,000 molecular weight on up to a milliom or more. I am not speaking at this point of haptens, those smaller molecules which can react with antibodies but cannot produce them; I refer now only to those substances which are antigenic, that is, which can produce antibodies as well as react with them. Most of the well-known and familiar antigens are proteins, but an increasing number have been found to be carbohydrate in nature—polysaccharides of various types—and there is some suspicion, not too well founded at present, that certain lipids can act as antigens. The most familiar antigens among the proteins are serum albumin and the other serum proteins, egg albumin, hemoglobin and hemocyanin, and casein. By contrast, certain proteins, such as gelatin, have long been regarded as nonantigenic, due perhaps to their deficiency in certain amino acids, such as tryptophane and tyrosine. However, the classical belief that gelatin is nonantigenic has recently been re-examined and we may have to change our views on this point.[2]

*Complex antigens*: Besides the simple proteins mentioned above, there are numerous complexes of proteins with other substances, such as nucleic acids, various carbohydrates, and lipids, which possess their own particular antigenic specificities. In addition to these naturally occurring protein complexes, there are a great variety of artificially altered protein complexes, first created by Obermayer and Pick[3] and tremendously extended by Landsteiner and his colleagues.[4] As you well know, these workers demonstrated that it is possible to bind chemically a number of relatively simple substances to proteins and, in so doing, to change the specificity of the protein. Thus, para-amino benzene arsenic acid (Atoxyl) can be bound by a diazo linkage to horse globulin. This yields an antigen which, when injected into rabbits, will produce a rabbit-globulin antibody that still reacts with horse globulin but also reacts strongly and specifically not only with the horse globulin-Atoxyl combination, but with a similar combination made with chicken globulin instead of horse globulin. This mechanism whereby new specificities may be created through the attachment of hapten groups to proteins has, of course, great potential significance for our understanding of the mechanism of sensitization to simple chemical compounds. Many of my colleagues in this audience are far more familiar with this concept than I am.

The specific properties of a protein antigen are well illustrated from another point of view by the behavior of diphtheria toxin when modified by treatment with formalin or ketene. A solution of diphtheria toxin protein has certain specific and measurable biologic and immunologic characteristics, to wit: (1) toxicity, (2) flocculation titer (''Lf''), (3) flocculation rate (''Kf''), (4) ability to coprecipitate with another toxoid and (5) antigenicity. If one adds about 0.3 to 0.4 per cent formalin to a solution of diphtheria toxin and incubates it at about 35 to 37° C., a series of changes occurs. First (but quite gradually) the solution loses its toxicity; a preparation of toxin which originally was capable of killing 3,000 guinea pigs per milliliter

should, after a few weeks of treatment, be totally innocuous to a guinea pig in a 5 ml. dose. Yet, if it has not been incubated too long or with too much formalin, its Lf and Kf titers will have remained unchanged. A little too much incubation or formalin will first affect the flocculation rate, which becomes slower. Further treatment will reduce the Lf titer to a varying degree. Prolonged treatment may completely destroy the ability to flocculate with antitoxin, yet the toxoid may still have the capacity to coprecipitate (to full titer) if blended with another toxoid. Somewhere during this process, though, antigenicity begins to decrease. Eventually it may be destroyed, although one occasionally obtains apparently fully antigenic toxoids which have no detectable flocculating ability.

The purpose of these observations is to bring out the fact that antigens have many properties and that each property may respond individually to various kinds of chemical or physical treatment. Thus, antigenicity may be altered quite independently of the other properties of an antigenic substance.

The tremendous differences in antigenicity of various proteins are not understood. Nor do we, as yet, understand such paradoxes as the unfortunately high efficiency of ragweed pollen as an antigen in man, in contrast to its poor performance in the guinea pig.

Of carbohydrate antigens we actually have even less knowledge. It is true that we know, from the classical studies on the pneumococcus capsular polysaccharide, that the removal of acetyl groups impaired the antigenicity of such preparations, at least in mice (although not in man). Related findings have been made recently by my associate, Dr. Landy,[5] and his colleagues, with the Vi polysaccharide antigen of typhoid bacillus.

*Polysaccharide antigens* are, in general, almost as widely distributed as protein antigens. They have particular significance as the determinant substances in the major blood group antigens, and they occupy a major role in a great variety of bacteria and other microbial agents, having turned out to be the prime antigens of many bacterial species. One of the peculiarities of polysaccharide antigens is that, in the two cases where their antigenicity has been fairly well studied (as with the pneumococcus by Heidelberger and the Vi typhoid antigen by Landy), a single dose of antigen appears to produce a more or less maximal antibody response which is persistent in most cases, and which cannot be further elevated by an additional stimulus. As will be apparent later, this is in marked contrast to the classical pattern of the response induced by protein antigens. It has been suggested that polysaccharide antigens are not readily metabolized by the host and therefore they remain in the antibody-forming cells for a long time, maintaining their antigenic stimulus out of all proportion to the usual response obtained with the more readily metabolized protein antigens.

One final comment on antigens has to do with our growing knowledge not only of the complexity, but also of the lability, of the antigenic patterns of certain infectious agents. This has been vividly brought home to us by the experience of the last ten years with the influenza viruses, particularly Type A.

Some have held[6] that influenza viruses possess a varied but limited set of antigenic components, any one or more of which may become predominant for a while and then shift, giving way to other members of the antigenic mosaic; others have pointed out[7] that entirely new antigens may appear from time to time, perhaps accompanied by equally marked changes in the virulence of influenza virus for man. As yet, it is not known which is true. A comparable, but even more dramatic, series of possibilities has been opened up by the work of Lederberg and his associates,[8] who have shown that the antigenic components of many members of the *Salmonella* group may be interchanged between species through the mechanism of bacterial virus transfer of the characteristic antigenic determinant. This opens up phenomenal possibilities of ceaseless, uncounted changes in antigenic pattern, and it somewhat dims our hopes that we can catch up with the antigenic patterns which we wish to bring under immunologic control. The implications of these findings for the allergies are not yet clear, but it becomes apparent that shifts and changes in allergic patterns may also be taking place from time to time, perhaps through mechanisms not yet recognized.

*Portals of entry:* How do antigens get into the host? This problem, of course, has been of primary interest to the allergist, and it is to the allergist that full credit must be given for having disproved the older teaching of the physiologists who claimed that unaltered proteins were not absorbed by the gastrointestinal tract. Walzer,[9] Ratner[10] and many other of our distinguished colleagues have established beyond a doubt that unaltered food proteins are absorbed through the intestines, and a classmate of mine once reversed his positive Schick test simply by drinking diphtheria antitoxin. Indeed, it may be assumed that most natural antigens enter the body through either the respiratory tract or the gastrointestinal tract, a fact which makes our parenteral injection experiments in rabbits and guinea pigs sometimes look just a trifle irrelevant! Our emphasis on the needle has been far too great, and the time is more than past when we should be engaged in extensive studies on the natural routes by which antigens gain entrance into man.

What about *persistence of antigens?* I have already mentioned the finding, well-confirmed in the case of pneumococcus polysaccharides[11] and indicated in certain other instances,[12] that under certain circumstances antigens may persist for long periods in the host cells. In the case of infectious diseases, it has long been suspected and recently it has been virtually proved,[13] that Brill-Zinsser's disease is the result of the persistence of viable typhus rickettsiae somewhere within the tissues of the recovered patient. Similarly, the Rocky Mountain spotted fever rickettsia has recently been shown by Parker and associates[14] to persist in the host for at least one year. Such findings as these undoubtedly add a great deal to our understanding of the lasting effect of antigens. However, it becomes difficult to believe that persistence of the viable antigen can explain, for example, the finding of high-titer yellow fever antibodies in persons who had the disease seventy-five years earlier.[15]

*The Antibody Response.*—Limiting most of our consideration to experiments based on parenteral inoculation, we have a certain amount of clear-cut knowledge of the pattern of response obtained. A single injection of an antigen

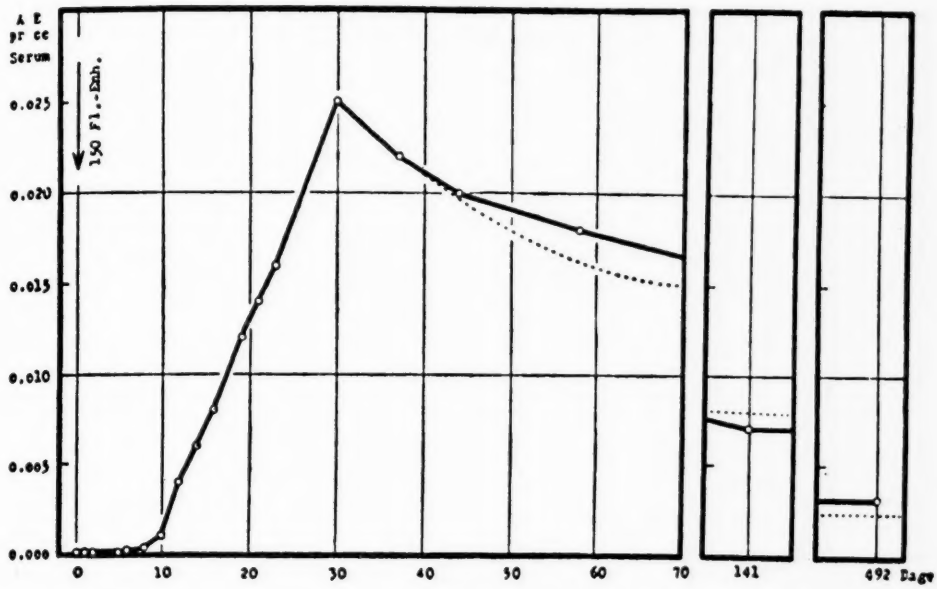


Fig. 1.—Effect of a single injection of 150 flocculating units of concentrated and purified anatoxin in a 4-year-old child without antitoxin prior to the injection. (From Jensen: Acta path. et microbiol. scandinav. **10**: 137, 1933.)

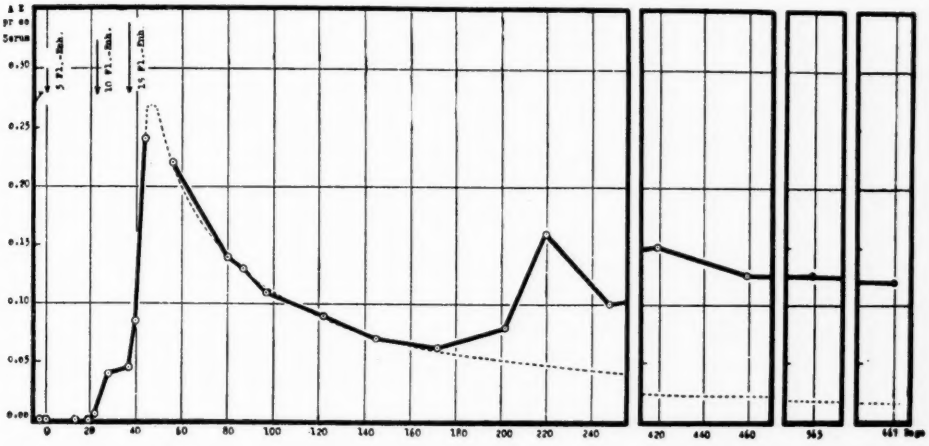


Fig. 2.—Effect of immunization with three injections (5, 10, and 15 flocculating units) of unconcentrated diphtheria toxoid in an 18-year-old girl (I. L.) who had no blood antitoxin prior to immunization. The marked secondary rise occurring about six months after immunization is probably due to natural, subclincal infection. Arrows show times when injections were given. (From Jensen: Acta path. et microbiol. scandinav. **10**: 137, 1933.)

will usually produce a measurable response in from two or three days to a month or more. (The reason for the great differences in the lag period is not yet clear.) The pattern of the antibody response is well illustrated in Figs.

1, 2, and 3, taken from a study published by Jensen[16] twenty years ago. Fig. 1 shows a girl without previous diphtheria antitoxin who received a very large dose of fluid toxoid. She first showed a slight antibody response after ten days. After thirty days it reached a peak, at a very low level of 0.025 unit of antitoxin. Thereafter, her titer gradually declined over the next one and one-half years. In another girl receiving only one-third this amount of antitoxin *in toto,* but in three separate doses, there was a rise to the much higher level of 0.25 unit. Her course is shown in Fig. 2. A third subject (Fig. 3) who already possessed antitoxin through natural exposure also received a large dose of toxoid; in this patient the rise began in six days and reached 100 units by the ninth day.

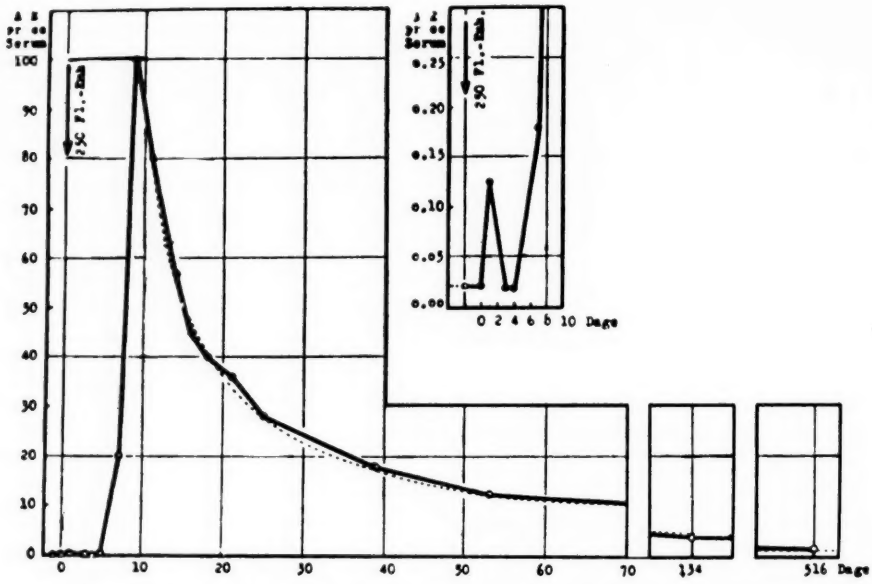


Fig. 3.—The effect of a single injection of 250 flocculating units of concentrated and purified diphtheria toxoid in a child (E. N., aged 10 years) with "natural" antitoxin (0.022 units per cubic centimeter of serum) prior to the injection. Insert shows details of response during first eight days. (From Jensen: Acta path. et microbiol. scandinav. **10**: 137, 1933.)

Of course, it should be borne in mind that these charts represent the familiar antibody response pattern which we sometimes are mistakenly tempted to regard as universal. A number of instances have been reported in which a booster dose of an antigen did not induce a rise in antibody. The best known of these studies were done with polysaccharide antigens[11] and rickettsial antigens.[17]

*The "booster" or third dose and spacing:* This important aspect of the immune response is seen in Fig. 4, which represents a generalization of such findings as those reported by Evans[18] for tetanus toxoid. Basic immunization alone will yield an antibody response such as that shown in curve *a*. If a booster dose is given after, say, about one year (see curve *b*), the result will be a tremendous, rapid, and persistent response so that two or three years later the

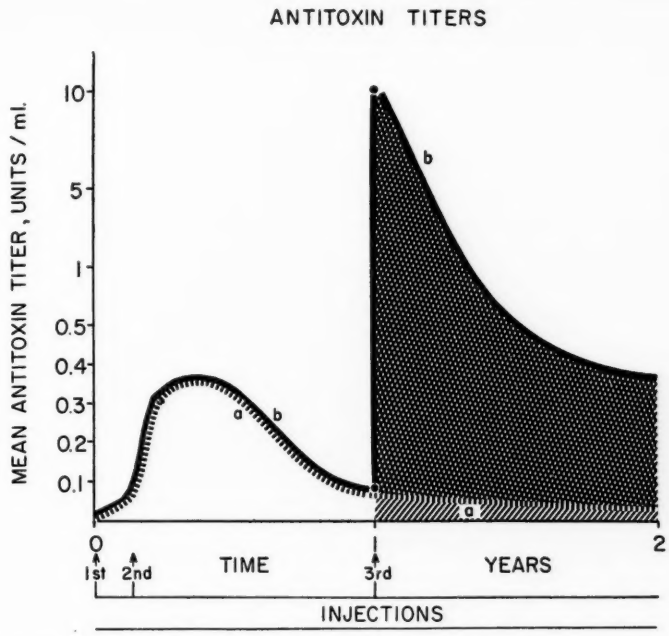


Fig. 4.—Antitoxin titers in the serum of subjects receiving either (a) two doses of toxoid or (b) two doses plus a third dose about one year after first injection. (Adapted from Evans: Lancet 2: 316, 1943.)

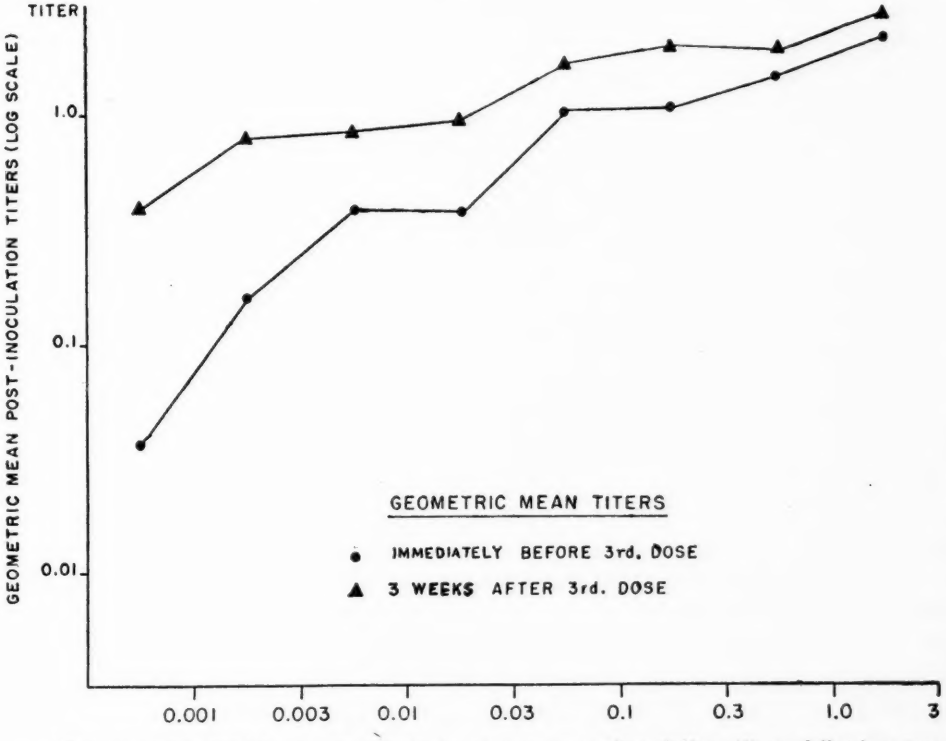


Fig. 5.—Relationship of postinoculation titers to preinoculation titers following two or three 1-Lf doses of adsorbed diphtheria toxoid. (From Edsall, Altman, and Gaspar: Am. J. Pub. Health 44: 1537, 1954.)

subject may still carry five or ten times the antibody level of the group not receiving this extra stimulus. We have obtained some rather similar data on diphtheria toxoid.

The uniformity of response is greatly affected by the number of doses administered. Fig. 5 shows the rather wide distribution of antibody levels which we found in a group of Navy recruits who at first were given two 1-Lf doses of diphtheria toxoid.[19] After these two doses, their antibody levels were distributed as shown by the line marked by black dots. The figures for the men who started with the lowest titers are given on the left and those for the men who started with the highest titers are given on the right. As would be

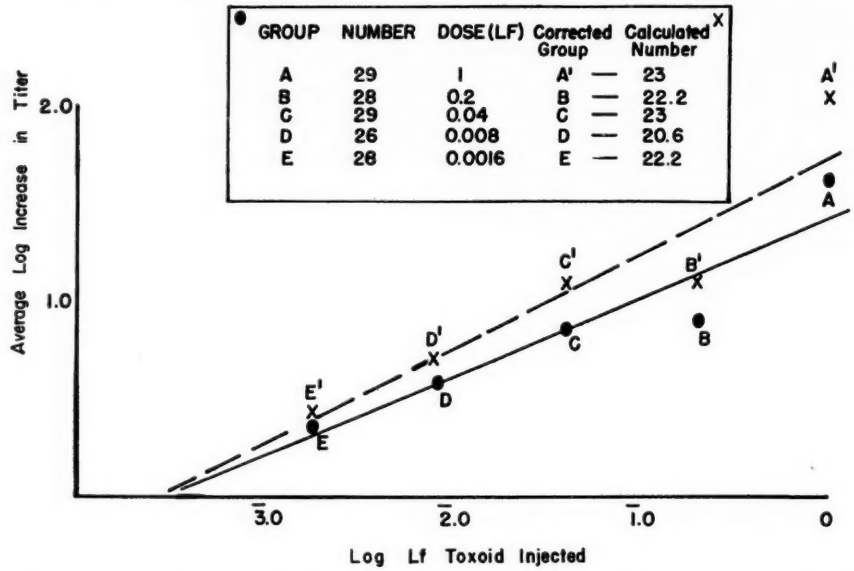


Fig. 6.—Relation of increase in diphtheria antitoxin titer to dose of toxoid administered. (From Edsall, Banton, and Wheeler: Am. J. Hyg. 53: 283, 1951.)

expected, the ones who started low did not rise as high as the ones who started higher, but they came up *relatively* more. Following a third dose, at six months, the whole group leveled off a good deal. The lowest ones came up the most, the highest ones rose the least, and the group wound up with a far more even distribution of antibody titers, as the line marked by the solid triangles indicates. There was not as wide a spread of antibody levels, nor as much individual difference, after that third, or booster, dose. Thus, it is often possible to achieve maximal and most uniform results with a given amount of antigen by proper spacing of the injections.

*Size of dose:* Let us consider the significance of the size of the dose in relation to the antibody response. In a group of 150 medical students, we gave five different doses of diphtheria toxoid and charted the average antibody increase.[20] Fig. 6 shows the consequences of giving 1 Lf, 0.2 Lf, one-fifth of that, and so forth (that is, decreasing the dose in fivefold steps from 1 Lf to 0.0016 Lf), in five different groups of medical students.

To convert this graph into plain language, what we found was that the change in antibody level produced was more or less equal to the square root of the change in the dose administered. In essence, this means that the antibody level obtained is not directly proportional to the amount of antigen given; for example, to increase the antibody response three times, it is necessary to increase the antigen dose about nine times. The converse, of course, is also true; reducing the antigen dose ninefold will reduce the antibody response only about threefold. This information is encouraging when one wishes to avoid reactions by cutting the antigen dose to the minimum compatible with an acceptable response.

Antigen dose, however, is not always determined just by a simple injection, and I think that this has an important bearing on our consideration of biologic phenomena. In infectious disease, antigen dose is determined largely by the multiplication of the agent, and variations in this factor can have some very interesting implications. This has been admirably brought out by Dr. Smadel[21] in the studies that he and his associates did on scrub typhus and its control with chloramphenicol. Fig. 7 shows, first, the normal duration of temperature and the gradual development of antibodies in untreated scrub typhus. However, if chloramphenicol is given to the usual patient seen on about the eighth or ninth day of the disease, the fever is rapidly aborted and the antibody response appears to be a little bit slower and perhaps not so high. Fig. 7 also shows what happens if one has the opportunity to give chloramphenicol as early as four to six days after onset of the disease. (This opportunity usually arises only in studies in infected volunteers.) Here the temperature drops abruptly, the antibody response starts up a bit later (but not much so), and the patient frequently relapses. A second dose of antibiotics cures the relapse and the patient normally remains well. Finally, when infected volunteers were given chloramphenicol as early as the second day of fever, the temperature was promptly aborted but there was almost always a relapse about a week or ten days later; and here you will note that no antibody appeared until quite late in the course of the disease. The point of these findings is that the production of antibodies definitely can be interrupted by an antibiotic. It seems that, without sufficient antigen over a long enough time, the host will not produce a normal amount of antibodies. This relationship of "dose" to antibody response in infectious disease has vast implications, and the principle simply awaits adequate circumstances and experimental techniques for carrying out basically similar studies in the field of allergy.

Next, I should like briefly to mention a question which has to do with the duration of the antibody response. Many of us have often wondered not so much at the sharp drop in antibody levels which I mentioned earlier, but at the fact that they usually do not disappear, even in a patient who is not continually exposed to the antigen in question.

Barr and Glenny,[22] in England, have gone one step further and shown in some studies with laboratory animals that, after a gradual fall, the antibody level will often start up again in what they call a "delayed or secondary rise."

This phenomenon is unexplained, but it seems to be one manifestation of the extraordinary ability of the host to maintain antibody levels, once it has been exposed to an antigen several times. These findings may contribute somewhat to our concept of the long-term character of the immune response. They do not

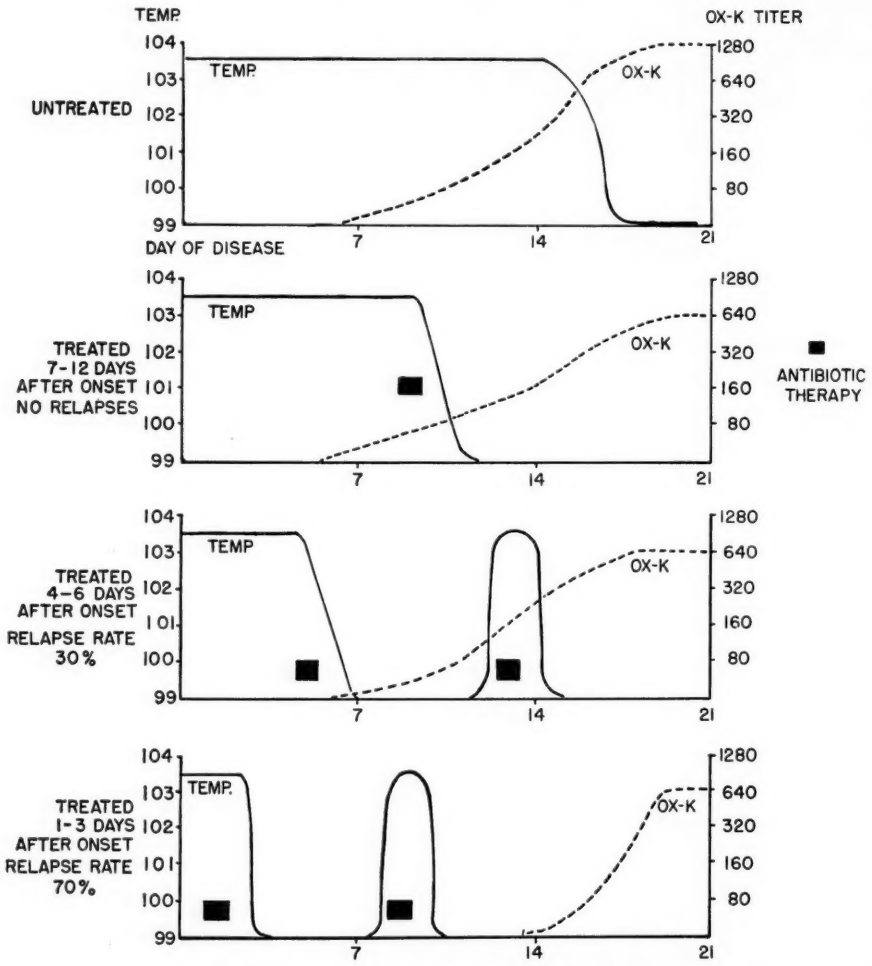


Fig. 7.—Diagrammatic representation of clinical and serologic response in patients with scrub typhus. (After Smadel: Am. J. Med. 17: 246, 1954.)

fit in with any theory that we have available at the moment, unless it should prove that there can be more persistence of antigen than we have yet been able to show. Nevertheless, there appear to be certain parallels in man—for example, Bojlen and Scheibel's[23] observations in children in Denmark, anywhere from three to nine years after their "basic" diphtheria immunization which consisted of two doses a month apart and a third dose one year later. They found that the antibody levels three years and nine years after the last toxoid injection were hardly distinguishable. Their studies showed that this persistence of antitoxin was not due to natural exposure to diphtheria infection.

My colleagues and I have obtained similar findings in tetanus antibody levels in 144 veterans seen anywhere from one to ten years after they had left military service and had their last tetanus toxoid.[24] The distribution of tetanus antitoxin titers observed is shown in Table I. The titers are shown in relation to the interval since the last dose of toxoid. In the distribution of values, from very low titers in the second column to the highest titers in the third column from the right, there is scarcely any difference, whether the interval since the last dose of toxoid was one year or ten years. This was a very happy finding, but it was a surprising one; it has since been independently confirmed by others.[25, 26] These findings all signify, I think, that we must recognize that, once an antibody mechanism is established, it may be far more persistent and far more sustained than our rabbit experiments used to lead us to believe.

TABLE I. TETANUS ANTITOXIN LEVEL BEFORE BOOSTER

| YEARS SINCE LAST IMMUNIZATION | TITERS OBSERVED (UNITS/ML.) | | | | | | | | GEOMETRIC MEAN (UNITS/ML.) |
|---|---|---|---|---|---|---|---|---|---|
| | 0.025 | 0.045 | 0.14 | 0.45 | 1.4 | 4.5 | 14 | TOTAL | |
| 10 | | 1 | 1 | 1 | | | | 3 | 0.14 |
| 9 | | 3 | 2 | 2 | | | | 7 | 0.12 |
| 8 | 2 | 3 | 5 | 3 | 4 | 8 | 1 | 26 | 0.59 |
| 7 | 3 | 4 | 12 | 13 | 3 | 4 | 2 | 41 | 0.32 |
| 6 | 4 | 3 | 4 | 3 | 4 | 4 | | 22 | 0.25 |
| 5 | 3 | 1 | 1 | 5 | | 3 | | 13 | 0.26 |
| 4 | 1 | 4 | 2 | 1 | 2 | 2 | | 12 | 0.24 |
| 3 | | 1 | 2 | 3 | | 1 | 1 | 8 | 0.51 |
| 2 | 1 | | 1 | | 1 | 1 | | 4 | 0.36 |
| 1 | | 1 | 2 | 2 | | 3 | | 8 | 0.60 |
| TOTAL | 14 | 21 | 32 | 33 | 14 | 26 | 4 | 144 | 0.34 |

(Adapted from Looney, Edsall, Ipsen, and Chasen: New England J. Med. 254: 6, 1956)

I should like to comment briefly on a phenomenon in which you may have been interested and with which some of you have worked in recent years; it is called, among other things, the "crowding-out" phenomenon. Many of us have wondered whether or not too many antigens given at one time will interfere with each other, and a few extreme examples of this have been found in experiments in rabbits and the like. Others (for example, Unger[27]) in recent years have tried to use this effect in suppressing the Rh response in Rh-negative pregnant mothers. Barr and her colleagues[28-31] have found evidence in guinea pigs that one antigen can interfere effectively with another under certain circumstances. Therefore, we have been quite concerned over the possible consequences in man of using combined antigens.

All I can report at this time is that some uncontrolled experiments of ours[19] in combined tetanus and diphtheria immunization showed no evidence of interference, and that Ipsen,[32] in Boston, has run controlled studies which showed no evidence of any interference, in this combination, by tetanus toxoid against diphtheria toxoid. Therefore, although the possibility of "crowding-out" exists, we have no substantial basis in man as yet either for worrying about it or for thinking that we can take advantage of it.*

*A recent paper by Chen and associates (*J. Immunol.* 77: 144, 1956) suggests that the "crowding-out" effect can be demonstrated in man.

*Host Factors.*—There is not time to discuss the various host factors which may affect the immune response. Several of them, either natural or artificial, have been brought out beautifully and strikingly earlier this morning, but I think it would be worth while to mention at least two or three which are of interest.

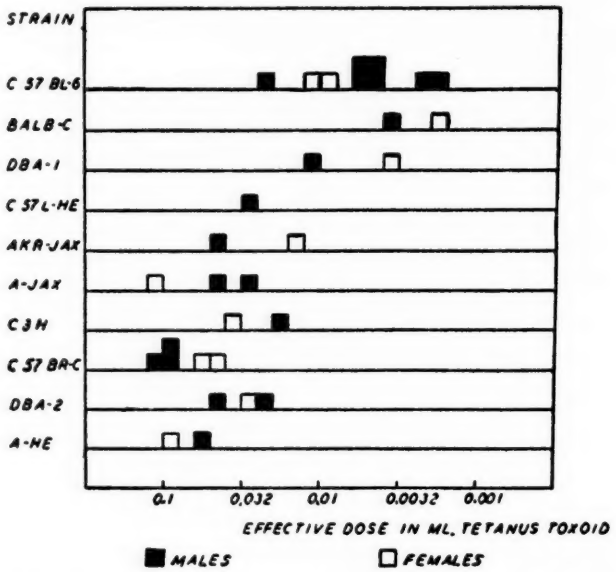


Fig. 8.—Relative immunizability of ten pure mouse strains, as determined by the dose of tetanus toxoid required to establish protection. Each small square represents one assay in twenty-one mice. (From Ipsen: J. Immunol. 72: 243, 1954.)

In support of the long-established belief that there are strain or tribe differences in immunizability, numerous examples have been shown in animals in the last few years. Fink and Quinn[33] have demonstrated genetic differences in the immune response in mice, and furthermore these differences seem to vary with different antigens. Ipsen[34] has made a very precise study in which he quantitated the ability of different strains of mice to respond to tetanus toxoid (Fig. 8). The protective dose of toxoid is shown in the abscissa. He used ten different strains of mice, each square representing a group of twenty mice. He found that several strains of mice were protected only if inoculated with about 0.1 ml. of tetanus toxoid, whereas other strains were protected equally well by as little as 0.003 ml. In contrast, there were no consistent differences in strain susceptibility to the toxin, according to a study which was run simply to show that the two factors were not parallel.

In England, Sang and Sobey[35] have done related studies in rabbits, showing, much as Fink and Quinn did, that the ability or inability to respond may not be a general phenomenon but that it may be quite selective with certain antigens. These findings can scarcely be surprising to allergists, who are more intimately familiar with hereditary factors in immunity than are the immunologists themselves.

*Age and the Immune Response.*—Finally, I want to speak of one field that interests me very much, namely, the significance of age in susceptibility and resistance. Despite numerous early reports to the contrary, it has now been well shown that antibody formation can occur even in very young infants. To be sure, Overman and his colleagues[36] (working with mice) have shown that there is an age prior to which the antibody-forming mechanism is quite ineffective. In very young mice (and only in such mice) they could produce a fatal encephalitis with mumps virus. As the ability to produce antibodies developed, the mice became resistant to this virus. If a virus of higher virulence was used, the development of resistance did not appear until later. More or less similar findings, in principle, have been shown for a neurotropic influenza virus studied in mice by O'Connor and Wagner,[37] but the nature of susceptibility, resistance, immunizability, and antibody response in infants and young children is not so simple as this would imply. Burnet,[38] in a fascinating paper entitled "Patterns of Disease in Childhood," has produced such data as those shown in Fig. 9. Here we see essentially three different patterns. The simplest is at the bottom: a progressively decreasing susceptibility to the lethal effects of influenza or respiratory disease, with a minimum at about age 11, and after that a progressive increase in susceptibility. Directly above the two bottom curves is the curve of influenza in 1918, showing the remarkable hypersusceptibility of young adults to the influenza of that year. Next above is shown the susceptibility of Americans to St. Louis encephalitis, with a minimum fatality rate in the teens. At the top is the curve for typhoid (in Australia), in which a pattern not unlike that of the 1918 influenza outbreak may be clearly seen. For us, I think that the principal lesson of these findings is that no one generalization can be made on this point. This, of course, is a familiar principle to allergists, who deal with food allergies in infants, with asthma and hay fever in young children, and with an increasing but changing pattern of dermal, pharyngeal, intestinal, vascular, and pulmonary disease as the human patient grows older. In this connection, there is much reason to be interested in the recent observation by Smith and Thomas,[39] that bacterial endotoxins may be less toxic for very young rabbits than they are for older ones. This finding may well turn out to be parallel and related to the frequent observation that certain infectious diseases are mild in children and more serve in adults. Analogies in the fields of allergy undoubtedly will occur to many of you.

One could speak of many other factors, such as nutrition, cortisone and other hormones, the influence of x-rays, and the intriguing but obscure finding that antibiotics may affect the immune response.[40, 41] However, I would like to close by remaining on the topic of age and re-emphasizing again the fourth dimension—time. From birth onward, we are subject to repeated insults, the effect of which must, in one way or another, be cumulative. You are all now familiar with the charts developed by many investigators, showing the rapid increase of poliomyelitis antibodies during childhood in various parts of the world[42, 43] (Fig. 10). A more recent example of this principle has been presented by Huebner and his associates,[44] who have shown that from the first year of

life all of us successively acquire antibodies to more and more of the particular group of recently discovered viruses—"adenoidal-pharyngeal-conjunctival viruses," as Huebner calls them—which he and Hilleman[45] have isolated.* By the time we are adults, all of us have met one or more strains of these viruses and one-half of us have responded to at least four or more of the six known strains. When we also consider poliomyelitis, the streptococci, the various

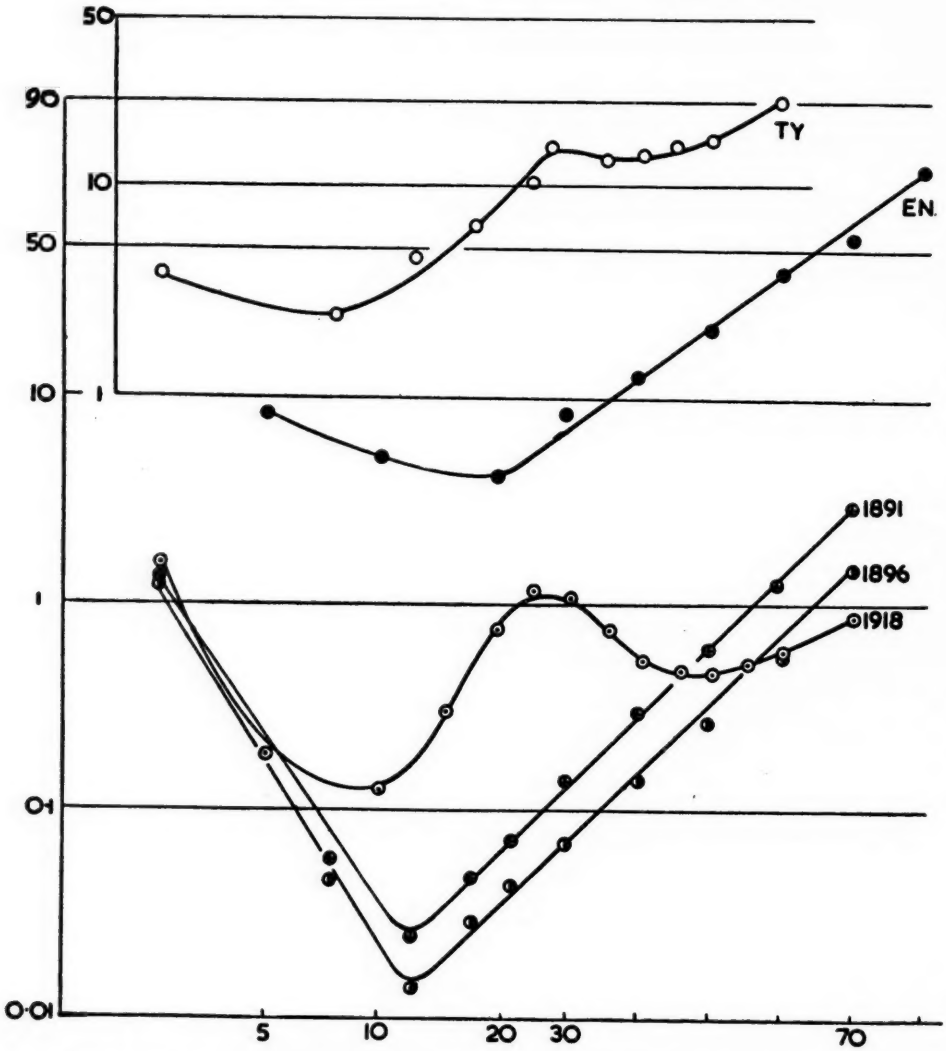


Fig. 9.—Case fatality rates by age for diseases predominantly of adult life. *Ordinates:* Logarithmic percentage scale. Time scale changes from logarithmic to linear at 25 years. *TY*, typhoid fever in Western Australia, 1912. *En*, St Louis encephalitis, 1933. Three bottom curves show respiratory deaths in England and Wales for the years shown. Data were obtained as follows: respiratory infections and influenza, 1891, 1896, 1918, Ministry of Health Report (1920); typhoid fever, Cumpston and McCallum (1927); St. Louis encephalitis, 1935, report on the St. Louis outbreak of encephalitis, U. S. Public Health Bulletin Number 214. (From Burnet: Australasian Ann. Med. **1**: 93, 1952.)

*These viruses are now called adenoviruses (Enders et al.: *Science* **124**: 119, 1956).

other cocci, the vast group of enteric bacteria, the fungi, and the numerous respiratory and miscellaneous viruses, it is a wonder that the gamma-globulin level of the blood is no higher than it is!

This discussion may seem to have wandered quite a bit from the immune response as we ordinarily conceive it, but there is a fundamental unity, to my mind, between infections, immunity, and allergy, as was very clearly brought

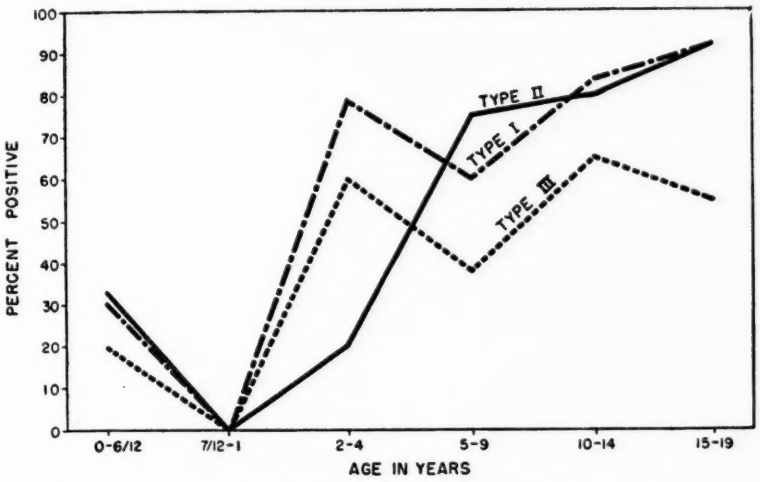


Fig. 10.—Poliomyelitis antibody distribution curves in inhabitants of Thomasville, Georgia, in 1952, as determined by mouse neutralization tests. (From Li and Schaeffer: J. Immunol. **72**: 123, 1954.)

out forty-five years ago by von Pirquet and Schick[46] in the final comments they made on serum sickness. Indeed, I think that immunology and allergy might be called parabiotic twins, in present-day experimental language, since they do share the same intellectual circulation.

My major point in this discussion is that I think that the more interchange we can bring about between the immunologists, the infectious disease investigators, and the allergists, the further each of the three of us will progress. I say "three" of us only from habit because, basically, I think that we are one.

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# SENSITIZATION WITH ORGAN SPECIFIC ANTIGENS AND THE MECHANISM OF ENHANCEMENT OF THE IMMUNE RESPONSES

JULES FREUND, M.D., NEW YORK, N. Y.

WHEN a suspension of kidney from an albino rat is injected into rabbits, antibodies develop which react with the antigens in the rat kidney.[1] If the rabbit serum bearing the specific antibodies is injected into rats, the recipient animals develop nephritis. This experimental disease is a useful tool for certain studies. From the standpoint of immunology, this phenomenon is not remarkable. The kidney suspension from the rat should be antigenic in a foreign species, namely, in the rabbit; the antibody should have an affinity to rat kidney, should combine with it, and should disturb the metabolic process in this organ. A suspension of *rat* kidney can be injected into the *rat* without producing antibody in the homologous species and without causing injury; that is, sensitization to the rat kidney.

During the past eight years, however, a group of new experimental diseases were discovered. The production of these diseases involves immunologic processes which probably are allergic in nature. One of the most interesting aspects of these experimental diseases is the fact that they can be produced by injecting homologous or autologous tissue suspensions to induce injury to certain organs or cells. For instance, one can inject a suspension of spermatozoa or testis from an individual guinea pig and produce testicular injury in the same guinea pig.[2] We may speak of allergic aspermatogenesis. This process is species specific since testicular material from foreign species, such as rabbit, sheep, or bull, does not induce testicular injury in the guinea pig. It is also possible to produce encephalomyelitis,[3] neuritis,[4] or uveitis[5] in guinea pigs or other experimental animals by injecting the suspensions of the respective tissues. These diseases can be produced at will and with a single injection, but the injection of the antigen by itself is not sufficient. It is a requisite for the production of these experimental diseases that the antigen to be injected be combined with potentiating agents. These potentiating agents are paraffin oil and killed acid-fast bacilli. By the use of these adjuvants, the animals' refractoriness to sensitization can be overcome.

Since these adjuvants play an essential role in the studies of allergy to tissue antigens, we will first discuss the potentiating agents.

The adjuvants have a long past and a short history. More than sixty years ago it was discovered that the addition of butter or oil, particularly

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paraffin oil, to acid-fast bacilli enhances the histologic lesions caused by acid-fast saprophytic bacilli.[6] About twenty years ago it was found[7] that sensitization to tuberculin in the guinea pig is augmented by incorporating tubercle bacilli into melted paraffin and by the injection of the combination into the subcutaneous tissue of guinea pigs. In the early thirties, following Saenz's[8] work, we found[9] that a very minute amount of killed tubercle bacilli in paraffin oil will produce a very high and permanent sensitization in the guinea pig. Sensitization to tuberculin is not related to circulating antibodies, and it was through a chance observation that we noted that not only are killed tubercle bacilli in paraffin oil very effective agents for sensitization to tuberculin, but that the addition of paraffin oil to killed tubercle bacilli will enhance and sustain the formation of complement-fixing antibodies to tubercle bacilli.

At this point, we took up some old experiments which had to do with an interesting problem in sensitization. Here we have to go back to another line of observations published during the early twenties. These observations dealt with the effect of tuberculous infection on immune response.

Lewis and Loomis[10] were studying experimental tuberculosis in highly inbred families of guinea pigs. In some control experiments they accidentally noted that tuberculosis increases the capacity of guinea pigs to form antibodies to various antigens. They have shown that if virulent, living tubercle bacilli are injected into the abdominal cavity of guinea pigs and five days later a suspension of sheep cells or killed typhoid bacilli is injected by the same route, antibody formation against these antigens will be more abundant than in nontuberculous guinea pigs treated the same way.

Dienes[11] confirmed and extended these observations. He found that tuberculosis alters sensitization to egg white or horse serum. Nontuberculous guinea pigs repeatedly injected with egg white react to egg white injected into the skin with an immediate type of reaction. This reaction appears within a few minutes to a half-hour. Redness and soft swelling are the characteristic reactions. The reaction diminishes within one or two days. This type of reaction can be transferred with serum to fresh guinea pigs. Sensitization to egg white may assume new characteristics in tuberculous guinea pigs. The reaction may be of the *tuberculin type*. It may appear only several hours after injection and it may last for two or three days. Induration and necrosis of the skin may be conspicuous, just as in the tuberculin reaction. It cannot be transferred with serum, but it is transferable with white cells. It must be emphasized that these two types of reactions to egg white may occur in combination in the tuberculous guinea pig. Moreover, very often only a small percentage of the tuberculous guinea pigs sensitized to egg white react to egg white with the tuberculin type of reaction, and most of them with the immediate type of skin reaction. Dienes also found that it is advantageous to inject the sensitizing antigen—for example, egg white or horse serum—into the tuberculous focus.

For several years we tried to repeat the experiments of Dienes, using killed tubercle bacilli rather than living ones. The results were irregular and discouraging. After we had made the observation that the addition of paraffin oil to killed tubercle bacilli enhances both sensitization and antibody formation, we took up the problem again. We[12] injected antigens, such as horse serum, diphtheria toxoid, or killed typhoid bacilli, into a focus produced by killed tubercle bacilli suspended in paraffin oil. We failed again. It occurred to us that the aqueous phase containing the antigen might be more effective if it were emulsified in paraffin oil containing killed tubercle bacilli. This can be accomplished easily with the aid of certain emulsifying agents. This type of preparation yielded satisfactory results. Later studies revealed that paraffin oil, even without the addition of mycobacteria, enhances and sustains antibody formation in a striking way. The addition of killed mycobacteria has, as a rule, an additional augmenting effect on antibody formation and it is a requisite for certain sensitization phenomena.

As mentioned before, when the guinea pig is injected repeatedly with well-known antigens, such as serum proteins or egg albumin, the guinea pig will react to the homologous antigen injected into the skin with an immediate reaction. This reaction may be of the evanescent type. It also may react with redness and soft swelling which last for about one day. Necrosis does not occur. If, however, the guinea pig receives the antigen in combination with paraffin oil and killed acid-fast bacilli, the skin reaction to the antigen will be different. It will be of the tuberculin type, just an in the tuberculous guinea pig. It will appear slowly and will last for about two or three days. Necrosis may be conspicuous. I hasten to add that these two types of reactions may occur in combination with one another.

The effect of mycobacteria on sensitization is very conspicuous with reference to sensitization with simple chemical compounds; for example, picryl chloride, as shown by Landsteiner and Chase.[13] This compound, dissolved in olive oil and repeatedly painted on the skin of guinea pigs, will sensitize the skin to a slight degree. When picrylated guinea pig red blood cell stromata are introduced under the skin it will hardly sensitize the guinea pig. However, if picryl chloride emulsified in paraffin oil which contains mycobacteria is injected under the skin, very intense and lasting sensitization will follow.

Even more striking is the effect of mycobacteria inducing allergic encephalitis. Repeated injections of a brain suspension combined with paraffin oil do not produce allergic encephalitis, but a single injection of the same emulsion containing killed mycobacteria will produce it in the majority of experimental animals. It is noteworthy that it is necessary to inject the mycobacteria together with the antigen. If the antigen and the mycobacteria are injected into separate sides, encephalitis will not occur.

For the production of allergic uveitis and neuritis, and for the production of allergic aspermatogenesis, mycobacteria are essential.

The mode of action of adjuvants has not been elucidated. Concerning the paraffin oil, we know that it makes the absorption of the antigen very prolonged. The paraffin oil attracts mononuclear cells to the antigen depot, and these mononuclear cells may favor antibody production. As for the role of mycobacteria, there are two views. One view is that the mycobacteria produce a cellular reaction (Fig. 1) which has an essential role in altering the type of hypersensitiveness. The other view[14] holds that the cellular re-

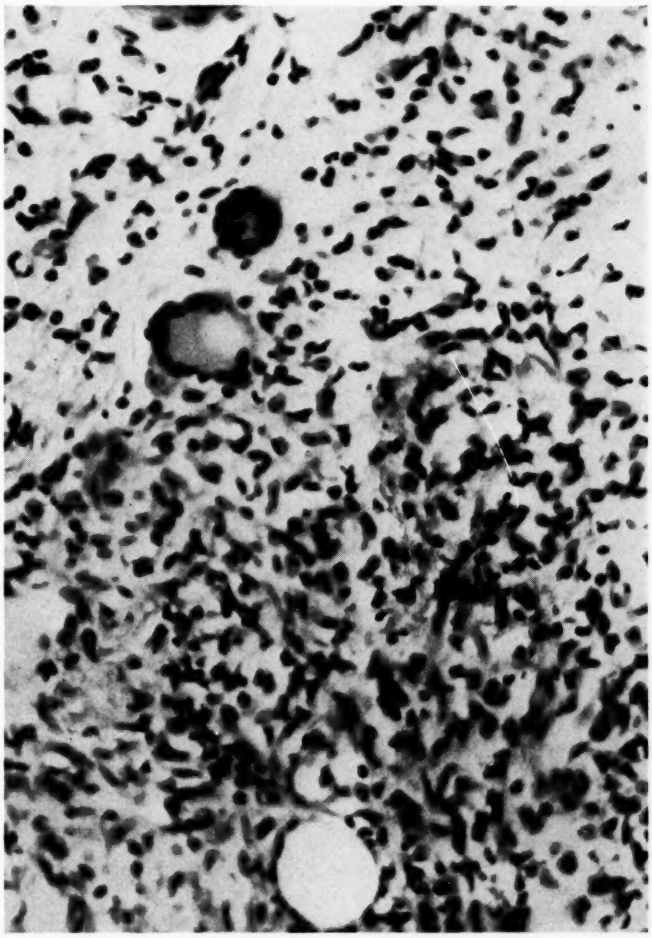

Fig. 1.—Reaction at site of injection of complete adjuvant with mycobacteria. Note oil droplets, particularly in a giant cell, and accumulation of mononuclear cells. (From Lipton and Freund: *J. Immunol.* **71**: 98-109, 1953.)

action to the mycobacteria is unimportant. This view assumes that the antigen combines with certain lipids of mycobacteria, the so-called wax, and that the combination of wax and antigen provokes the tuberculin type of immune response. I believe in the first view, namely, that the role of mycobacteria is the creation of a certain cellular reaction about the antigen and that the cellular reaction favors the development of allergy.

In discussing sensitization with organ antigens, we are concerned with four experimental diseases: encephalomyelitis, neuritis, uveitis, and aspermatogenesis.

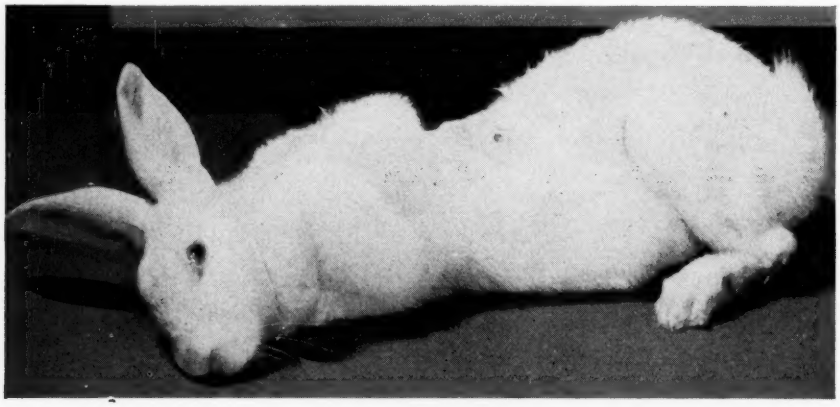

Fig. 2.—Flaccid paralysis.

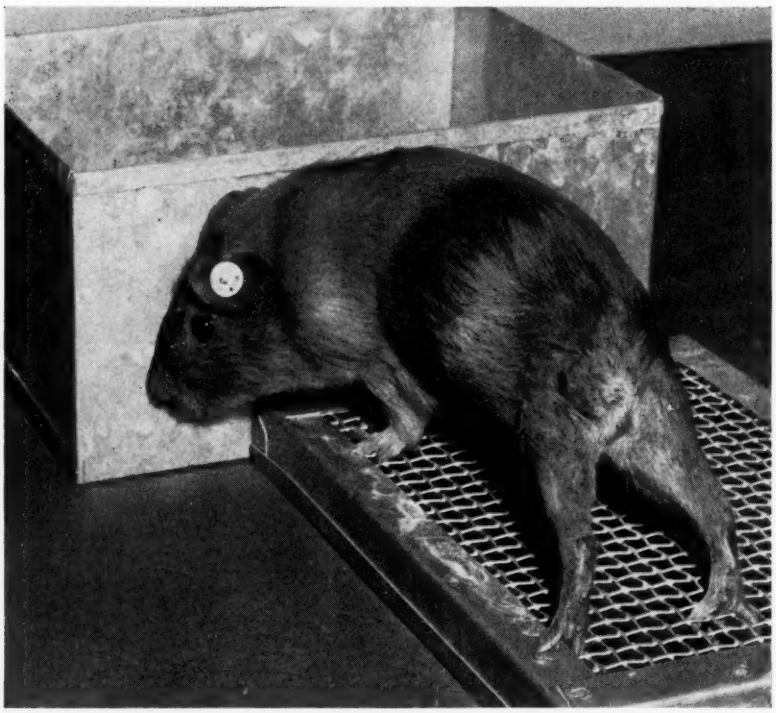

Fig. 3.—Spastic paralysis (rare). (From Freund, Lipton, and Morrison: *Arch. Path.* **50**: 108-121, 1950.)

*Allergic Encephalomyelitis.*—It was assumed many years ago that certain types of encephalitides occurring in man are allergic in nature. Examples are encephalitis after measles or after vaccination against smallpox and so-called paralytic accidents following vaccination against rabies. Furthermore,

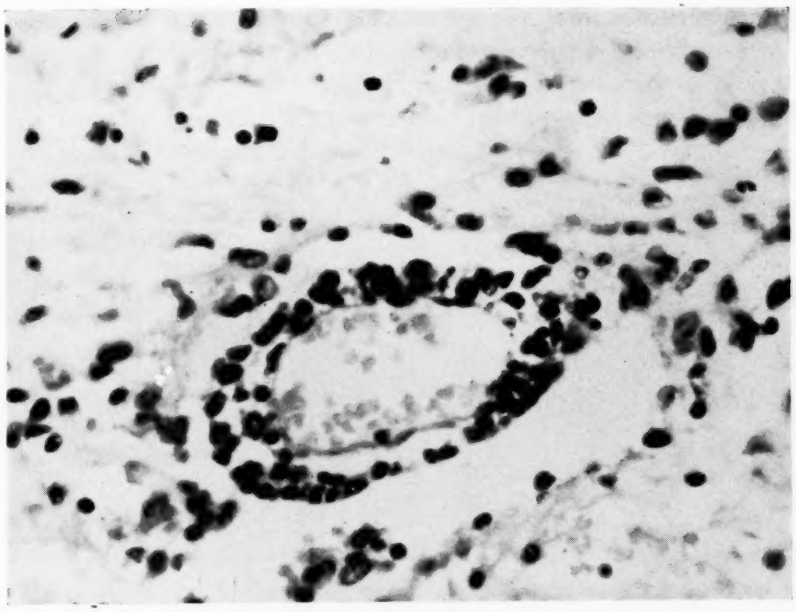

Fig. 4.—Perivascular cuffing and spread of inflammatory cells in brain of rat. (From Lipton and Freund: *J. Immunol.* **71**: 98-109, 1953.)

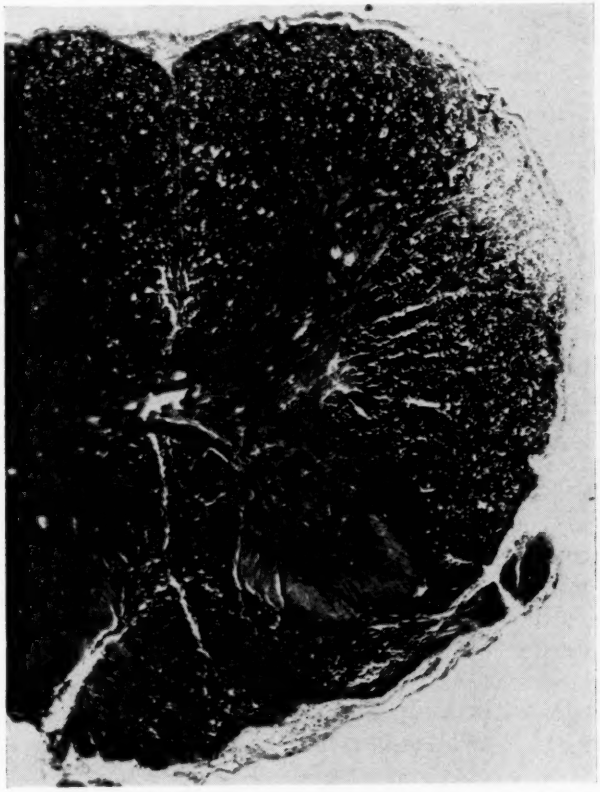

Fig. 5.—Demyelination in spinal cord of guinea pig. (Spielmeyer stain.) (From Freund, Lipton, and Morrison: *Arch. Path.* **50**: 108-121, 1950.)

it was suggested that multiple sclerosis may be of allergic origin. More than twenty years ago Rivers and associates[3a] showed that if monkeys are given from 30 to 100 injections of monkey or rabbit brain suspension, a few of the injected monkeys develop encephalomyelitis. Recently it has been shown that a single cutaneous injection of brain from the monkey or other vertebrate

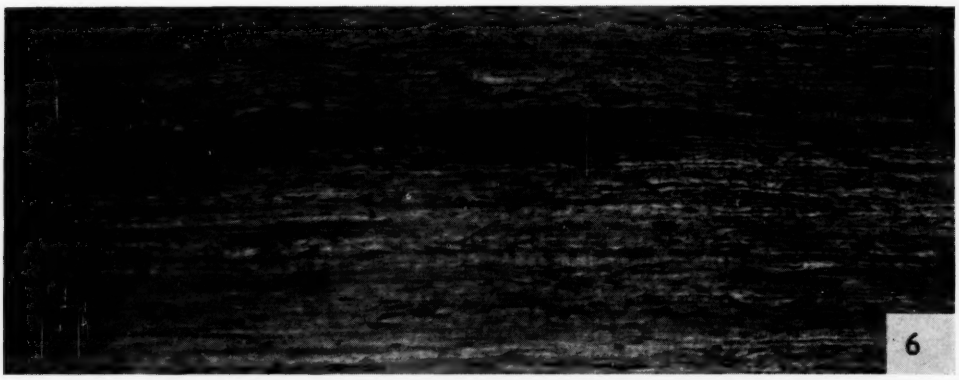


Fig. 6.—Peripheral neuritis in rabbit. (From Waksman and Adams: *J. Exper. Med.* **100**: 451, 1955.)

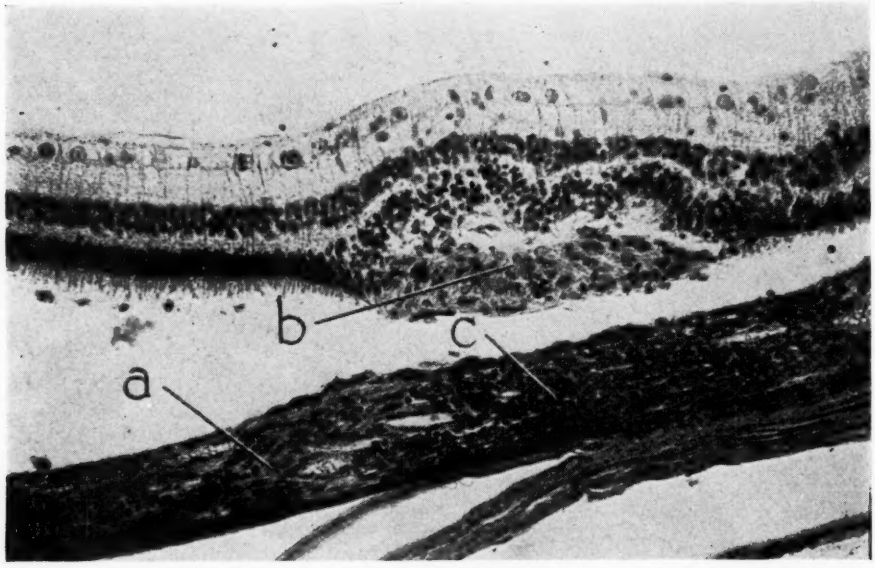


Fig. 7.—Uveitis. (Collins; cf. reference 5.) *a*, Area of epithelioid cells in center of nodule in choroid; *b*, proliferation of pigment in epithelium; *c*, area of lymphocytes in choroidal nodule. (From Collins: *Am. J. Ophth.* **36**: 150, 1953.)

species (but not fish) will produce encephalomyelitis, provided that the brain suspension to be injected is combined with complete adjuvants, that is, paraffin oil and killed acid-fast bacteria.[3b, c, d] If the brain suspension is from a heterologous species, the injected animal develops encephalomyelitis and also skin sensitization to the brain suspension.

*Allergic Neuritis.*—It is of interest that the animals with allergic encephalomyelitis do not show peripheral neuritis. However, as shown by Waksman and Adams,[4] the injection of a suspension of peripheral nerve, such as the sciatic nerve, combined with complete adjuvants induces neuritis in rabbits.

Fig. 8.—Allergic aspermatogenesis in guinea pig.

*Uveitis.*—The experiments on allergic uveitis were stimulated by the assumption that sympathetic ophthalmia may be an allergic response to the antigens of the uvea of a diseased eye. Until recently, efforts to produce uveitis in the experimental animal by repeated injections of suspensions of uvea failed. A few years ago Collins,[5] of Columbia University College of Physicians and Surgeons, showed that a suspension of guinea pig or rabbit uvea combined with paraffin oil and killed mycobactria injected subcutaneously will produce uveitis in the guinea pig. The uveitis may progress to panophthalmitis. The histologic section of the eyes can be superimposed on sections from human sympathetic uveitis.

*Allergic Aspermatogenesis.*—The experimental diseases just discussed—encephalomyelitis, neuritis, and uveitis—are induced by injecting suspensions of the respective organs. Allergic encephalomyelitis can also be produced by an *extract* of the brain.[15] Allergic aspermatogenesis, however, can be induced by injecting a single cell type, namely, spermatozoa. While the first three diseases can be provoked by using the suspension of the respective organs from

Fig. 9.—Normal guinea pig testis. (From Freund, Lipton, and Thompson: *J. Exper. Med.* 97: 711-726, 1953.)

other species, aspermatogenesis occurs only when homologous or autologous testicular material is injected. Thus, the antigenic material in aspermatogenesis is not only organ specific but also species specific.

It is of interest that aspermatogenesis can be produced by a purified extract of testis.[16] The antigenic material is highly active, since a single

injection of a few thousandths of a milligram is sufficient. The antigen remains active after boiling, and is resistant to proteolytic enzymes. It is associated with a carbohydrate and with amino acids.

Guinea pigs injected with testicular extract develop anaphylactic sensitization. Typical anaphylactic shock can be elicited by the intravenous injection of extract in sensitized guinea pigs. Likewise, an intense skin reaction will follow the injection of a small amount of antigenic material into the skin.

Anaphylactic sensitization to an organ extract from homologous species is unexpected, although it has been shown that guinea pigs can be made anaphylactic to the proteins from guinea pig crystalline lens. However, there is a difference between the two phenomena. Testicular extract represents material from the cells of the testis, but the proteins of the lens are in a free fluid and may be considered secretions. Secretion products, such as casein and thyroxin, have a very limited species specificity and may be antigenic in the homologous species.

In conclusion, the following remarks may be justified:

1. The administration of an antigen in paraffin oil emulsion enhances and sustains antibody formation.

2. The use of adjuvants may find application in prophylactic immunization and preseasonal treatment of allergic patients.

3. The addition of killed mycobacteria to the emulsion modifies and potentiates allergic sensitization. It is a means of demonstrating the potential allergenic nature of tissue and other antigens.

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## Discussion

DR. FEINBERG.—The presentation by Dr. Freund holds much of importance for us at present and in the future. He really has presented two subjects which are of vital interest to us—(1) the possible effect of adjuvants in desensitization and (2) the potential influence of auto-antigens as a cause of disease in an organ such as he has demonstrated, which might possibly apply also to such other organs as we are interested in, that is, the lung or the bronchi. I think that there should be some discussion or questions on this presentation.

DR. MERRILL CHASE.—I think that we should be keeping clearly in mind the fact that in paraffin oil and in the tubercle bacillus, there are two different adjuvants. In the experiments that Dr. Loveless has done with pollen extract there is no tubercle bacillus, whereas in these very fine tissue effects that Dr. Freund has done the tubercle bacillus is necessarily added to the paraffin oil, so that we have to distinguish between two different things, and even a third, when they are both put together, for the tubercle bacillus in saline is relatively ineffective and paraffin oil, for Dr. Freund's tissue damage, is ineffective. However, it is quite adequate for enhancing the antigenicity of certain viral and particulate materials in the guinea pig, where repository effects and not cellular changes are required.

DR. GEOFFREY EDSALL.—I have just one brief accent. We had some comment this morning on the necessity for re-examining traditions, and I think that Dr. Freund and the others who have worked with isosensitivity have thoroughly blasted the concept of *horror autotoxicus*—the idea that the body could not react to something not contained within itself. It is high time that the term *horror autotoxicus* was relegated to the shelves of medical history and that we no longer are fooled by any belief that that is a general principle.

DR. FREUND.—Dr. Chase spoke of the separation of the effects of oil and killed tubercle bacilli and the combination of both. The oil has the effect of preserving the antigen, to some extent, and setting up many depots and producing favorable cellular reaction to antibody formation. The killed tubercle bacillus could have two effects. One is that it could

create a kind of cellular reaction, which is really a necessary condition for a certain type of sensitization. In a skin section at the site of injection of paraffin oil and killed tubercle bacilli, you see many cells. They are not polymorphonuclear leukocytes, but they are epithelioid cells. You also see giant cells, in the cytoplasm of which are oil droplets containing some killed tubercle bacilli. I believe that this kind of local reaction has to do with the type of sensitization we achieved.

The other possible effect is that killed tubercle bacilli contain a wax which favors sensitization when the wax combines with the antigen. I do not hold with that idea. The reasons would be a little too complicated to go into, but one of the reasons why I do not hold with it is that aspermatogenesis, uveitis, or encephalomyelitis cannot be produced by using wax instead of tubercle bacilli.

With reference to Dr. Loveless' observations, I believe that they depend on the fact that there are some antibodies, as discovered by Dr. Cooke and studied extensively by Dr. Loveless and Dr. Sherman, which favor not sensitization, but desensitization, although possibly they are produced against the same antigen, which is able to sensitize. That would be my explanation.

# THE MECHANISM OF SENSITIZATION

MERRILL CHASE, PH.D.,* NEW YORK, N. Y.

THE question of sensitization is as broad as immunology, dealing as it does with evidences of physiologically altered balances and special patterns of response by tissues subjected to antigenic or allergenic stimuli. Further, this topic allotted to me is couched in exceedingly blunt fashion, quite as though I —or others who could be quoted—comprehended the subtle ways of Mother Nature. This is, obviously, a framework question. It will be used periodically in your sessions for years to come.

Newer elements of structure, nevertheless, can be welded to the frame. For illustration, reference will be made to experimentation conducted primarily in laboratory animals. I ask your further indulgence in allowing me to choose as models the patterns observed in drug sensitization of the guinea pig. Ostensibly, such an approach is remote from the interests of the practicing allergist and suggests, rather, those of the veterinarian. No route, however, that leads into the area of the patterns of response by tissues is to be neglected.

In certain respects, indeed, I believe that more of basic immunology is revealed by application of sensitizing chemicals than by the more customary choice of, for example, ragweed extract or native proteins. When one sensitizes with protein materials and watches with a shrewd eye, as Louis Dienes has done, the very first stage is seen to be a transient induction of delayed-type sensitivity. This event is overshadowed almost at once by the appearance of circulating antibodies and immediate-type sensitivity. In consequence, the observations of Dienes have been largely ignored. But this initial immunologic event is not transient in drug sensitization, where the determinant group comes from without and the carrier protein is autologous. At the same time, an excess of antigen gives rise, sooner or later, to circulating antibody, so that one who works with drugs is not hindered from studying protein-type sensitivity arising at the same time.

The first experimental evidence dealing with the modus operandi of simple chemical allergens may fairly be said to date from the time when Karl Landsteiner and John Jacobs chose compounds of the dinitro- or trinitro-substituted benzene series for injection into experimental animals, since a wide variety of basically similar chemicals was afforded in the possible permutations of similar groupings attached to the benzene ring. Seventeen compounds of those that were possible came into their hands, and it was determined which were positive and which were negative with respect to the capacity to sensitize guinea pigs. It turned out that those compounds that were positive had one common characteristic, a lability of one of the attached chemical radicals under conditions

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*Associate Member, Rockefeller Institute for Medical Research, New York City.

favoring mild hydrolysis. Because of this circumstance, the determinant part of the allergenic chemical could unite with basic groups of tissues and soluble proteins. In contrast to the sensitizing capacity of chemicals that possessed a high or perceptibly large reaction constant in the presence of sodium methylate, for example, as a hydrolytic agent, chemicals possessing very low reaction constants failed to induce sensitization in guinea pigs.

The amounts finally chosen for purposes of experimental sensitization were quite small. They totaled 15 to 50 mcg. administered into the skin in single doses of about 2.5 mcg. per injection site. Alternatively, it is often possible to sensitize the skin by repeated superficial contacts. The existence of the hypersensitive state may be determined by making contact reactions on the skin with the allergenic chemical itself being applied in solution, as in an oil as a convenient vehicle, or one may inject a conjugate that has been prepared in vitro by causing the chemical allergen to react with soluble protein.

It is not evident, as yet, why the skin is the necessary site for administration of simple chemical allergens. A basic problem remains despite the demonstrated correlation between chemical lability and allergenic capacity, for if, in imitation of an auto-antigen in which the body supplies the protein part of the antigen for reaction with the chemical, we combine an allergenic chemical with body protein (serum protein, keratin, red blood cells) in vitro, the reaction product will fail to induce sensitization.

Herman Eisen and his colleagues at New York University have extended these early studies of Landsteiner and Jacobs. Only one area of this excellent work will be mentioned here. Eisen has examined the sensitizing and eliciting properties of 2,4-dinitro-substituted benzene rings having, in labile position 1, a variety of reactive substituents (fluorine, chlorine, bromine, $SO_3$, SCl, SCN) and he has employed chemical allergens marked with radiocarbon or radiosulfur for purposes of tracing in vivo. An attempt was made to determine the combining sites in the skin with which the chemical allergens united. Accordingly, the skin of shorn guinea pigs was sprayed with various compounds from the list of eliciting chemicals just cited and the skin was taken and hydrolyzed on the following day, at which time the 2:4 dinitrophenyl groupings were found to be completely in the dermis. It turned out that certain of the elicitors, namely, those having halogen (fluorine, chlorine, or bromine) at position 1, had combined in vivo with the end amino group of the basic amino acid lysine and that others, namely, those having $SO_3$, SCl, or SCN at position 1, had combined with the sulfur grouping of cysteine. Accordingly, we conclude that different kinds of reaction products can occur between skin and elicitor, depending upon the chemical properties of the entire structure, even when it is a question of 2:4 dinitrophenyl groupings becoming attached to protein. Similarly, in coupling in vitro, Eisen has shown that the point of coupling of allergen with protein may depend in certain instances upon the pH at which the reaction is conducted.

The sensitization that is induced by chemical allergens is predominantly of the so-called "delayed type"; it is measured by the allergic inflammation that commences some hours after contact of the skin with the chemical and reaches

its peak at twenty to forty-eight hours. There may be present, in addition, circulating antibodies accompanied in some measure by a reactivity of the "early" or "evanescent" type. The conjugates made in the laboratory between allergen and protein give rise only to the latter variety of reaction, witnessed in the guinea pig by development of an anaphylactic state. Accordingly, it is not to be thought that the protein conjugates are inert antigenically; they simply fail to give rise to delayed-type contact hypersensitivity. In this article, unless specifically stated otherwise, the word "sensitization" will refer to delayed-type contact hypersensitivity.

Foremost among the principles deduced from attempts to engender a state of hypersensitivity toward drugs and simple chemicals is the role played by irritation and local cell accumulation. Repeated exposure to "bland" concentrations will, in all probability, effect sensitization only in those who are hereditarily most susceptible. One can imagine, for instance, that in the *Schierseife,* once used so widely in Germany, it was the sodium hydroxide and sodium carbonate content that helped the process of sensitization, and that physically the splinters in the furrier's sawdust used during the treatment of furs may assist in the induction of hypersensitivity to paraphenylenediamine employed as dyestuff. We in the laboratory know that various ways of irritating are conducive, but the irritation should be relatively mild in degree. We can, for example, select concentrations of an allergenic chemical to produce the proper degree of primary irritation upon either injection or topical application; we can establish the degree of irritation deliberately, using a topical application of dilute cantharidin or organic peroxide to prepare skin for the application of one of the less active chemical allergens; or we can excite a specific allergic reaction due to some previously established hypersensitivity and apply some new allergenic chemical on the resulting bed of mild irritation and cell accumulation. In the same way, the burned area to which butesin picrate is applied may be electively prepared for the induction of hypersensitivity to picric acid, or the inflamed and fissured toe may be ripe for the onset of sensitization following a topical application of penicillin salve.

The role of heredity has been investigated in guinea pigs with respect to their susceptibility to experimental sensitization. Hereditary "preparedness" plays a fascinating and highly significant role, determining the degree to which the animal can be made sensitive to a particular allergen. The role of heredity, large as it is, does not function as an absolute control, and appears much less dominant than that which produces the prominent familial patterns observed in reaginic-type sensitivity of the human being.

One special means of inducing experimental sensitization, applicable not only to chemicals but even to protein-chemical conjugates, is to employ the tubercle bacillus (or other *Mycobacteria*) with its peculiar property of creating focal accumulations of special types of cells. If dead tubercle bacilli (or certain of their products) are combined with a preformed conjugate, we can readily sensitize guinea pigs to exhibit typical contact-type reactivity toward the simple chemical. (The function of the mycobacterial substance, however, is not understood.)

A surprising and technically useful result has been achieved from inducing contact-type hypersensitivity by means of protein-chemical conjugates in conjunction with *Mycobacteria*. When animals so sensitized are subjected to several contact tests with the simple chemical itself, the degree of hypersensitivity increases exquisitely in a manner not otherwise seen. Instead of reacting to contact with allergen in a limiting concentration of about 1:500 in solution in an oil vehicle, such individuals may react to a concentration of 1:50,000. In this peculiar and special case, one can work with guinea pigs that essentially are as sensitive as human beings are wont to become.

Another facet in experimental sensitization is the possibility of preparing guinea pigs so that they are specifically refractory to sensitization. When certain known allergens are administered to nonsensitive guinea pigs by feeding, in such a manner that the skin of the muzzle is uncontaminated by the chemical, a resistance to later deliberate sensitization with the same chemical is induced. Certain initial routes of administration other than per os give variable degrees of protection; feeding induces the state of resistance most regularly. The resistance is manifest for more than a year after cessation of feeding, probably due to some persistence of the chemical grouping combined with tissues at sites that are effective, for unknown reasons, in deviating some necessary event among those required for effecting sensitization. Evidence to date shows that such chemical groupings as may persist in the tissues are not able to absorb antibodies from administered specific antiserum. Most probably there is an actual immunogenic unresponsiveness following the feedings. Fed and resistant animals undergo typical sensitization with respect to nonrelated allergens. The phenomenon recalls the resistance to the production of antipneumococcal antibody by mice that have been given pneumococcal polysaccharide of the same specific type. Ostensibly, there are differences between the two kinds of resistance. Pneumomoccal polysaccharide may be given by several routes, the quantity is large enough to absorb specific antibody injected into the mouse, and the polysaccharide may be reisolated several years later.

It would be possible, then, to conceive of experimental sensitization as a process in which susceptibility within a susceptible species is subjected to at least two limiting factors, namely, the certain limitations imposed by heredity and the possible limitations that may have been established by prior contacts with the same allergenic chemical. Both factors would tend to establish a "ceiling" on the degree of sensitivity that can be induced in a particular individual. Despite the fact that Grolnick, in imitating some of the feeding experiments in man with extract of *Krameria*, has not been able to demonstrate an analogous blockade toward later sensitization, I am rather of the opinion that the principle supersedes by far the methods employed to date in testing the idea and that there exists a general biologic principle of broad implication.

Apart from the questions just raised, the other points cited above—the favoring relationship of mild inflammation and cellular accumulation at the site of contact with the chemical incitant, the apparent necessity for combination of chemical allergen with the patient's body substances, and penetrance directly into epidermis or through natural pathways such as hair follicles and

sweat glands—are all undoubted elements in the mechanism of sensitization of the contact dermatitis type. These elements have to do chiefly with receptivity on the part of the subject himself. The major role played by the special chemical properties of individual drugs and chemicals has not been stressed. Some can sensitize nearly every person; others give rise to hypersensitivity only sporadically. We can only guess at what happens between "treatment" and ultimate sensitization. The newer attacks with radiotracer allergens undoubtedly will lead to some degree of resolution of the fundamental processes involved. Something has been learned, however, regarding alterations in the host that are accompaniments of sensitization.

Some years ago, in attempting to transfer various products from sensitized animals to normal ones, we found that collections of white cells could impose on normal guinea pigs the specific sensitivity that had been induced in guinea pigs of the donor group. We have not been successful in attempts to cross the species barrier, that is, to sensitize other species by means of the white cells of the guinea pig. These experiences with the transfer of drug hypersensitivity were first confirmed by Haxthausen, who employed thymic cells from donor guinea pigs rendered hypersensitive to dinitrochlorobenzene. We ourselves transfer intact, washed, white cells secured from lymph nodes, spleens, or peritoneal exudates which have been obtained after injection of paraffin oil. But the first studies of Jeter, Tremaine, and Seebohm, using guinea pig cells that have been disintegrated by sonic vibration, appear to give positive, even though weak, effects. The technique is not sufficiently elaborated, I take it, but from it we may expect new progress in analysis of the effect.

The durability of the effect is of variable degree. Much effort has been put into the study of its duration. Suffice it to say that, under proper conditions of transfer and in circumstances in which special hazards are circumvented, the procedure of testing for hypersensitivity can lead to active sensitization. An animal can be rendered hypersensitive for many months. Close examination shows that there are probably two stages following cell transfer: one event occurs after a few hours to two days and may well be over in five to six days; a second event may occur between five and eight days after transfer, likewise due to some material that is provided by the transferred cells. The latter event appears to be responsible for effecting nearly permanent conversion to the hypersensitive state. A paper to be presented later in these sessions will deal in greater detail with this study.

Dr. R. L. Baer and his co-workers, who have been making first attempts at studying the technique of cell transfer from persons suffering from allergic dermatitides, have concluded that cell transfer is a special method of inducing an active sensitization. According to our own reasoning, their studies have dealt chiefly with the second stage of the two mentioned above.

Bacterial allergies also are transferrable by means of white cells. Tuberculin hypersensitivity and *Brucella* hypersensitivity can be so transferred between guinea pigs. By the same means, Lawrence has transferred tuberculin and streptococcal sensitivities of the delayed type between human beings.

Furthermore, Lawrence has been able to transfer sensitivity with the use of lysed rather than intact cells, and indeed it was this finding that stimulated the studies of Jeter and associates with guinea pig cells. Lawrence has shown that the active material in the lysate is resistant to enzymes which destroy both desoxyribose nucleic acid (DNA) and ribose nucleic acid (RNA) within the cellular material.

Freund has employed a technique that appears to depend for its successful outcome on the principle of cell transfer, namely, the transfer of allergic encephalomyelitis between rats by means of parabiosis. Previous attempts to effect transfer between guinea pigs by means of isolated cells had failed.

Other studies could be mentioned on the use of cell transfer. One significant finding was that of Wesslén, who was able to transfer tuberculin hypersensitivity between guinea pigs by means of the lymphocytes taken in quite "pure" suspension from the thoracic duct. This study probably provides the most direct evidence available regarding the significant cell type, although the correlation between active suspensions and numbers of lymphocytes has been apparent for a long time.

As I mentioned at the beginning, in experimental drug allergy in which the cutaneous reactivity is definitely of the delayed type we often meet with circulating antibodies. In order to detect these antibodies, we must possess soluble protein-drug conjugates. Such conjugates can be used in studies both in vitro and in vivo. The chemistry of the compound used for sensitization and the intensity and duration of the sensitizing injections determine the concentration; usually we find antibody in trace amounts only. When antibody production occurs in higher concentration, it is seen to determine a separate and evanescent type of cutaneous reactivity that may add to and blend with the delayed type of reactivity.

When one is conscious of entering the realm of circulating antibody and available antigen, he feels himself to be on surer ground. The facts of immunology and of immunochemistry are at his disposal, but actually these facts are far fewer than we would wish, particularly as regards the genesis of antibody.

We know that antigen can persist within tissues, at least in trace amounts, for many months, for McMaster has studied the fate of a conjugate antigen made of bovine gamma globulin and a blue dye present as a prosthetic group. Crampton and Haurowitz have found that antigen introduced into the blood stream enters into the microsomal fraction of liver cells but, very shortly thereafter, becomes located within mitochondria and within nuclei. The latter conclusion has received confirmation by Coons, with intracellular antigen being traced by means of fluorescing antibody. Accordingly, antigen could be viewed in vivo as a sort of intranuclear inclusion.

As regards the production of antibody by tissues, many workers have made in vitro studies by culturing splenic tissue taken from rabbits a few days after injection of antigen; antibody appears as tissue is cultured. Parker made shrewd observations; more recently, Roberts and White have made contributions, while London has added radioactive amino acid to tissue that is

elaborating antibody in vitro and has found incorporation of the amino acid in the antibody that appears. Antibody has appeared in vivo following the transplantation of tissue from antigenically stimulated animals. Topley used minced spleen. Quite recently, Oakley et al. have shown the appearance of diphtheria antitoxin after transferring fatty tissue from animals that were producing this antibody. Also under way are experiments by Stoner in which a local production of antibody occurs within the eye of the mouse following implantation of certain tissues. Perhaps, even quite probably, the cell in these tissues that is responsible for the elaboration of antibody is the plasma cell, as the work of Fagraeus would indicate. Plasma cells are to be found in the spleen of the antigenically stimulated rabbit some few days after antigen is provided. Newer findings, however, would seem to implicate the lymphocyte, as well, in the story of antibody production, for it happens that the same cell suspensions that transfer the delayed type of dermal reactivity are capable of giving rise to the small amounts of circulating antibody that are found in the hypersensitive donors of the cells.

The latter finding was originally quite surprising. Preformed antibody present within the cells could be excluded, as extracts and dead cells were both inactive. The amount of antibody is not large, but it is of significant magnitude and it is specific. For proper study, therefore, more cells should be transferred than are necessary for the establishment of dermal sensitization. The same cell sources that we have cited before in effecting transfer of dermal hypersensitivity are effective in causing the appearance of antibody. Antibody may be found as early as twenty-four hours after the transfer of cells, but usually it becomes detectable between forty-eight and seventy-two hours later; it accumulates gradually, reaching a peak around the fifth to ninth days in the guinea pig and then falling away with an apparent half-life of perhaps seven days. It turned out that the transfer of antibody by cells did not require the preparation of dermally hypersensitive donors; one had only to prepare guinea pigs to produce antibody to various common antigens, and cell transfer would lead to the appearance of circulating antibody.

Now all evidence at hand leads to but a single conclusion: the circulating antibody that appears after cell transfer from hypersensitive guinea pigs is *not* the explanation for the dermal hypersensitivity that arises also. The two effects arising from the injection of white cells are independent of one another. The rapidity of the establishment of hypersensitivity and the onset and accumulation of antibody are different. The hypersensitive state can remain for many months; the antibody disappears and at a later specific "recall" injection the animal behaves as one that is experiencing the antigen for the first time. Further, and perhaps most conclusively, animals especially prepared so that they are producing antibody of the same specificity as that which appears *along with* dermal hypersensitivity, but prepared so that they *are not sensitized* to exhibit delayed-type contact reactions, yield cells that cause the appearance of antibody in the circulation of the recipient but fail entirely to induce any dermal contact-type sensitivity in the recipient.

The dichotomy of effects evidently depends solely on the way in which the animal intended as donor for cell transfer has been treated. If both dermal hypersensitivity and antibody production have been imposed upon it, the cells of the lymph nodes, for example, will transfer both effects; however, if it has been prepared to produce antibody only, its cells will give rise only to antibody. Evidently, it requires little extrapolation, if any, to conclude that dermal contact-type hypersensitivity depends upon a variant immunologic pathway.

The phenomenon of the appearance of antibody following cell transfer from antigenically stimulated rabbits has been studied by Harris, who used bacterial antigens, and by Wager and Chase, who chose diphtheria toxoid as antigen; it has been studied by Wesslén in Sweden with cells (said to be 99 per cent lymphocytes) from thoracic duct lymph. In man, Walzer and Glaser have used white cells from blood in initial experiments in securing human reagins from this cell source.

Perhaps it would be well, in concluding, to reverse the very first sentence and to say instead that immunology is as broad as the question of sensitization.

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## Discussion

DR. WILLIAM B. SHERMAN.—As a practicing doctor among these immunologists, I should like to make one comment with regard to the interesting factor that Dr. Chase has described—the manner in which oral administration of allergenic substances to the guinea pig inhibits the development of sensitization.

The type of work that Dr. Chase has described is practically the whole basis of our understanding of clinical drug allergy, and yet our difficulties in applying it are well illustrated by the fact that we are dealing largely with the results of oral administration of drugs in producing sensitization in human beings. Yet, in the guinea pig, the effect seems to be exactly the reverse.

DR. CHASE.—Dr. Sherman, I do not consider this to be a basic conflict, as you evidently do. There is no model or prototype of a method of sensitization of guinea pigs by the oral route, thus far. Indeed, it might be difficult for the allergist to prescribe a method for effecting sensitization of the human being by means of drugs administered per os. Infrequency of effect commonly means that the necessary factors are not under our full control or understanding. As regards the specific "blockade" of the guinea pig by feeding, it should be pointed out that we have dealt with relatively few different compounds, but the mode of handling these compounds has been well known to us. Once we became convinced that "blocked" animals existed (and the initial observations were *not* made on animals that had been fed), we conducted seventeen experiments to learn how to accomplish the result at will; from these experiments, feeding was hit upon quite unexpectedly. Have so many types of trial been made in the human being? It may be rather early to look for the common denominators between the species, but please do not think that our experiments were undertaken in any way to develop a method for "protecting" man from exposure to drugs. We are searching for principles. Practical applications, if any can be made, must follow.

DR. SHERMAN.—I mention it merely as an illustration of the difficulties of applying these brilliant experimental results in guinea pigs to our clinical problems.

DR. GEOFFREY EDSALL.—Mr. Chairman, I would just like to reinforce that difficulty and that problem. I have been faced with that same problem in trying to work out the pattern of immunity to typhoid fever. Our guiding philosophy has been the statement made by Metchnikoff forty years ago, when people used guinea pigs instead of mice to play around with *Salmonella typhosa,* that between typhoid peritonitis in the guinea pig and typhoid fever in man, there was only one thing in common—the name of the microbe.

DR. CHASE.—That calls for an answer. I will defend these experiments in both the way in which they were carried out and the purpose that leads us to go ahead with them. In the case of the typhoid, we have an organism that is adapted to the human species and causes the particular disease only in that species. Is that not so?

DR. EDSALL.—That is true also in the chimp.

DR. CHASE.—All right. We'll put in the chimp. But still we do not have typhoid fever in the mouse. We can give it only an endotoxic death. Correct?

DR. EDSALL.—And septicemia.

DR. CHASE.—All right; septicemia and endotoxic death. In the case of dinitrochlorobenzene, we have specific sensitization of both species, don't we?

DR. EDSALL.—That's right.

DR. CHASE.—We have knowledge also of other chemicals that effect sensitization in both these species similarly. Of course, one could simply follow the advice that I received from a leading allergist and dermatologist upon the death of Dr. Landsteiner, with whom I was so long associated; he assured me that careful histologic examination of reactions in guinea pig skin would be different from those of the human being and that I might as well drop the problem of studying drugs in the guinea pig. But I replied to him, and now say again: "Let us take just one species and study it thoroughly enough to learn what the events are during sensitization. Then, if analogies between species do exist, let them come to light." In the instance of cell transfer, which started with the guinea pig that had been sensitized with drugs that sensitize man, I opine that the experiments with man have been profitable, regardless of present or future interpretation.

DR. SHERMAN.—Dr. Chase, I believe our understanding of drug allergy is fundamentally based on the type of work that you are describing. I do not mean to minimize it in the least.

DR. EDSALL.—I would like to say one thing. My remarks were intended in your defense, Dr. Chase, the point being that, because we cannot reproduce this in man in exactly the way we want to, I merely wanted to point out that that is no barrier. It will be done some other way.

DR. CHASE.—You are probably right. I think so.

DR. RUDOLPH BAER.—I certainly agree with what Dr. Sherman just said, namely, that one cannot immediately transfer the conclusions from the experimental animal to human beings. On the other hand, I think that some of our experiments have been giving a very excellent illustration of the point that Dr. Chase just made, namely, that we just have to keep on trying to apply the principles which have been discovered in experimental animals and see whether they also hold in the human being.

For instance, in our attempts to produce passive transfer of exanthematous sensitivity in human beings by means of white cells from sensitive donors injected into nonsensitive recipients, we were unable to produce temporary sensitivity. On the other hand, we discovered a phenomenon in the human being, namely, that about 10 or 12 per cent of the recipients of white cells whom we had used in our passive transfer experiments, while they had not become passively sensitive, gave evidence that they probably had become actively sensitive. When we spoke to Dr. Chase about it, we found it had already been discovered in experimental animals by the Harrises. In other words, these white cells of human beings, apparently similar to the white cells of sensitive animals, had contained some factor that made these human beings more susceptible to active sensitization.

Incidentally, Dr. Haxthausen in Copenhagen, who worked with attempted passive transfers of exanthematous sensitivity, also found that one of the subjects in his much smaller series became actively sensitive, so that this phenomenon, for which we were not looking at the time, was found to exist probably in human beings as well, while the passive transfer was not achieved. But, of course, the qualitative relationships in the experiments done by Haxthausen and by our group are entirely different from those used by Dr. Chase in experimental animals, and I think that we will just have to keep on trying more.

DR. CHASE.—As long as you keep on trying, yes.

DR. FEINBERG.—In view of the arduous discussion about the limitation of species in experiments, I believe that all the other papers to follow, it should be said, are limited to only the species that they discuss and no inferences should be drawn otherwise.

# NONPRECIPITATING ANTIBODY AND CIRCULATING ANTIBODY-ANTIGEN COMPLEX

LUDWIG A. STERNBERGER, MAJOR, MC, USA*

THE antibodies appearing after immunization can usually be characterized by precipitation, complement fixation, or agglutination, but the antibodies encountered in clinical allergy have so far escaped detection by serologic means.

It appeared to us that the differences in behavior of the antibodies which form after immunization and of the antibodies found in clinical allergy may have some bearing on the physiology of clinical allergy as compared to immunity.

It is generally assumed that the precipitating antibody appearing after immunization combines with the immunizing antigen and rapidly eliminates it from circulation. It has been known since the classic experiments of von Dungern[1] and it has recently been confirmed by Dixon and his group[2, 3] that the appearance of antibody in circulation follows the disappearance of antigen. Free antigen and its homologous precipitating antibody apparently do not coexist in a single system.[4] On the other hand, most theories on antibody formation require the constant presence of antigen in order to explain continued formation of antibody. How could we otherwise understand the persistence of antibody in some allergic patients, even though they had no contact with the antigen for years?

If allergy were due to nonprecipitating antibody, it would be possible for the complex between antibody and antigen not to precipitate, and it would be feasible that antigen and antibody could exist together in circulation. However, nonprecipitating antibody has not been detected so far in allergic patients.

The methods used for demonstration of nonprecipitating antibody in rabbits depend on the binding capacity of the antibody with the antigen and on the subsequent removal of such nonprecipitating antibody from the system by an independent procedure. For example, in Heidelberger's[5] method for the determination of nonprecipitating antibody, precipitating antibody is reacted with antigen in the presence of nonprecipitating antibody. A precipitate due to precipitating antibody and antigen forms, and nonprecipitating antibody is estimated by any increase in the expected amount of precipitate due to binding of the nonprecipitating antibody. This method obviously depends on the binding capacity of nonprecipitating antibody with the antigen in the precipitate. We shall show that this holds true for only a fraction of nonprecipitating antibodies.

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*Present address: Army Chemical Center, Maryland.

Again, nonagglutinating Rh antibody can be detected by its binding capacity with Rh-positive cells in the Coombs test. Here the cells that have bound nonagglutinating Rh antibody are agglutinated by a precipitating antiserum to the absorbed human antibody globulin.

Our own studies on nonprecipitating antibody were prompted by the finding that soluble antibody-antigen complex exists in the circulation of immunized rabbits in large amounts.[6] Demonstration of soluble antibody-antigen complex depended on its conversion to precipitating antibody-antigen complex in vitro (Fig. 1). The serum containing such complex is brought to pH 12.6 at 1° C. It is left at this pH and temperature for six minutes. It is then neutralized and, upon neutralization and incubation at 1° C., a precipitate forms. This precipitate forms in some immune sera, but it does not form in normal sera. It comes out without any further addition of either antibody or antigen. Its amount can be estimated by nitrogen determination, and its specificity is demonstrated by the fact that it dissolves in an excess of the specific antigen but does not dissolve in nonspecific protein. Of course, this is a behavior which we expect from any specific antibody-antigen precipitate.

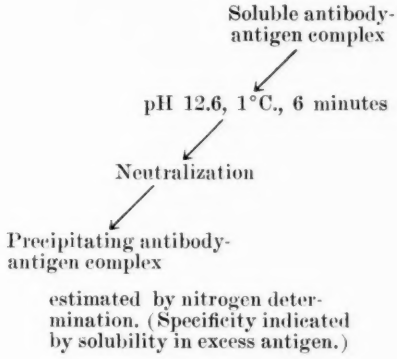
Soluble antibody-antigen complex
pH 12.6, 1°C., 6 minutes
Neutralization
Precipitating antibody-antigen complex
estimated by nitrogen determination. (Specificity indicated by solubility in excess antigen.)

Fig. 1.

We called this procedure "dissociation procedure," because we have previously applied similar treatments to the dissociation of precipitating antibody and antigen,[7] to dissociation of antibody-antigen-complement complex,[6] and to the dissociation of thrombin antithrombin complex of serum.[8] Applied to immune sera, the procedure apparently converts soluble antibody-antigen complex to a precipitating antibody-antigen complex.

Fig. 2 shows the immune response of a rabbit receiving a single dose of 1 Gm. per kilogram of bovine plasma gamma globulin. Antigen disappeared rapidly after inoculation. No antigen was found on the twelfth day. Free precipitating antibody was first shown on the twelfth day. Only small amounts of free antibody formed, and they persisted until the thirty-third day. The new entity, soluble circulating antibody-antigen complex was first detected on the seventh day, at the time when free antigen still was present in circulation. It

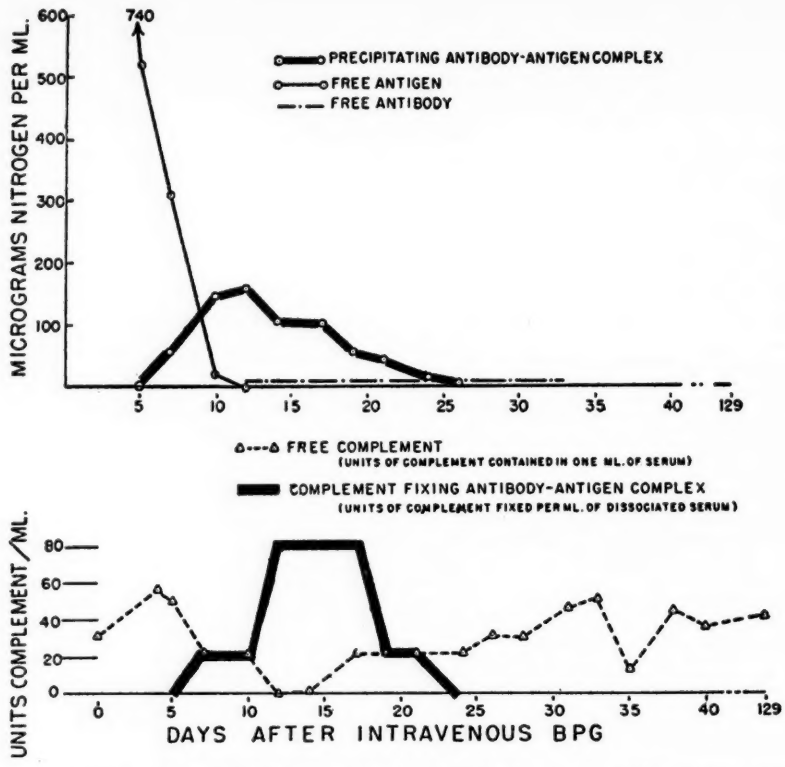


Fig. 2.—Formation of precipitating and complement-fixing antibody-antigen complex in vitro from nonprecipitating and noncomplement-fixing circulating antibody-antigen complex following injection of a single large dose of bovine plasma gamma globulin. (From Sternberger: *J. Exper. Med.* **98**: 451, 1953.)

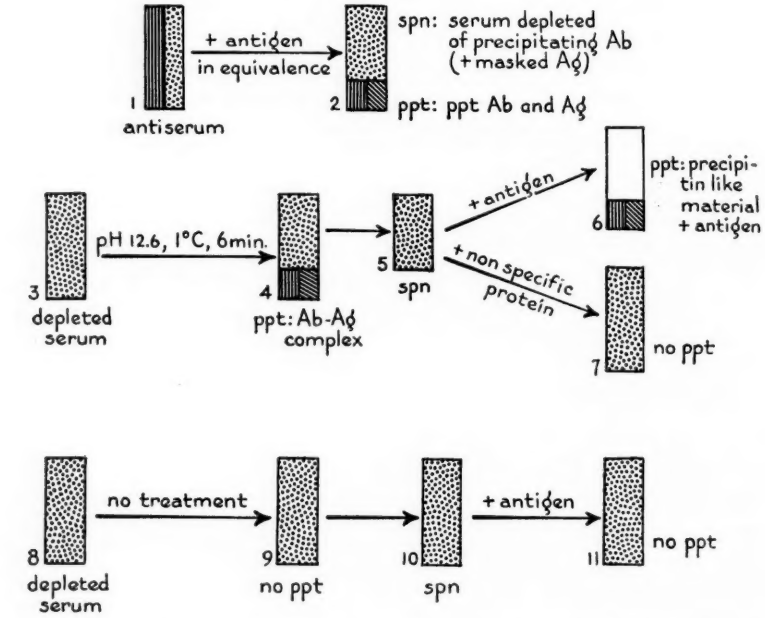


Fig. 3.—Schematic representation of estimation of nonprecipitating antibody in serum following depletion of precipitating antibody, dissociation and removal of antibody-antigen complex. (From Sternberger, Feinberg, and Clarke: *J. Exper. Med.* **103**: 523, 1956.)

reached its maximum on the twelfth day, at a time when antigen had disappeared from circulation and antibody made its appearance. No antibody-antigen complex persisted beyond the twenty-fourth day, although a trace of free precipitating antibody still was found in the serum.

We expect a maximum reaction between antibody and antigen to occur at the time antigen disappears from circulation and antibody makes its appearance. It is interesting that, just at this time, antibody-antigen complex had reached its maximum. It is puzzling, though, that such large amounts of antibody and antigen can coexist as a complex in circulation without precipitating.

In order to study this, we have focused our attention first on the antibody part of the complex and later on the antigen part. The findings will suggest that the antibody of the complex is a nonprecipitating antibody and that the antigen part may be either the original antigen injected or a metabolic breakdown product of such antigen.

It appeared to us that if antibody-antigen complex is due to nonprecipitating antibody, there also may be present in the serum free nonprecipitating antibody beyond the amount necessary to bind the antigen in the complex.

TABLE I

| ANTIGEN NITROGEN ADDED (MICROGRAMS) | NITROGEN PRECIPITATED PER MILLILITER UNDILUTED SERUM (MICROGRAMS) | | | |
|---|---|---|---|---|
| | SERUM A | | SERUM B | |
| | UNDISSOCIATED | DISSOCIATED | UNDISSOCIATED | DISSOCIATED |
| *Crystalline Bovine Plasma Albumin* | | | | |
| 4 | 0 | 105 | 0 | 78 |
| 7 | 0 | 153 | 0 | |
| 14 | 18 | 281 | 0 | 142 |
| 18 | 24 | 293 | 0 | 142 |
| 36 | 24 | 349 | 0 | 172 |
| 72 | 12 | 349 | 0 | 228 |
| 144 | 0 | 349 | 0 | 228 |
| 180 | | 308 | 0 | 222 |
| *Crystalline Egg Albumin* | | | | |
| 18 | 0 | 0 | 0 | 0 |

Fig. 3 illustrates the method we used for determination of nonprecipitating antibody. In the first step, the precipitating antibody is removed from the serum by a sufficient amount of antigen. A precipitate forms, as shown in tube No. 2, which is composed of precipitating antibody and antigen. The supernate from tube 2 is a serum depleted of precipitating antibody. This supernate is shown in tube 3 and it is treated by the dissociation procedure. Following incubation, antibody-antigen complex precipitates. The supernate from this precipitate in tube 4 is shown in tube 5; when antigen is added to this supernate, again a precipitate forms. This precipitate forms upon the addition of specific antigen, as shown in tube 6, but when nonspecific protein is added no precipitate forms, as represented in tube 7. Undissociated controls show that these precipitates form only upon dissociation. Without treatment, no antibody-antigen complex comes down, as shown in tube 9; if, then, specific antigen is added to this material, again no precipitate forms, as shown in tube 11.

Table I shows application of the procedure to a pool of serum obtained after intravenous injection of crystalline bovine plasma albumin. Serum A was depleted of precipitating antibody by repeated additions of small amounts of antigen, and the precipitates formed were removed. To a portion of this depleted serum, varying amounts of antigen were added, and only trace amounts of precipitate formed. This is shown in the second column of Table I. Another portion of the serum was treated by the dissociation procedure and antibody-antigen complex was allowed to separate. To the resulting supernate there were added varying quantities of crystalline bovine plasma albumin and large amounts of precipitate came down, as shown by the third column of Table I.

Serum B was treated in the same way as serum A but, besides being depleted of precipitating antibody, it was also depleted of the monovalent antibody of Heidelberger. The serum contained 18 mcg. of such antibody nitrogen. To one portion of this doubly depleted serum, varying quantities of bovine plasma albumin were added and absolutely no precipitate formed. Another portion of this serum was dissociated and antibody-antigen complex was allowed to separate. To the resulting supernate varying quantities of bovine plasma albumin were added, and again large amounts of precipitate appeared.

TABLE II

| CRYSTALLINE BOVINE PLASMA ALBUMIN NITROGEN ADDED (MICROGRAMS) | RESIDUAL CRYSTALLINE BOVINE PLASMA ALBUMIN NITROGEN #1 IN SUPERNATE (MICROGRAMS) | |
|---|---|---|
| | UNDISSOCIATED | DISSOCIATED |
| 4 | 3 | 0 |
| 7 | 6 | |
| 14 | 12 | 5 |
| 18 | 18 | 10 |
| 36 | 45 | 25 |
| 72 | 82 | 67 |
| 144 | 175 | 150 |
| 180 | | 175 |

These data are typical of experiments showing that in dissociated serum there is a material which can be precipitated specifically by antigen, while nondissociated serum fails to form precipitates. The dissociation procedure apparently converts a nonprecipitating antibody in serum to a precipitin-like material. To qualify as such, however, it should not only combine with the specific antigen, but it should also be able to remove such antigen from solution.

Table II shows the antigen-removal capacities of the precipitates of serum B in Table I. The supernates from these precipitates were analyzed for their antigen contents by the method of Heidelberger and Kendall,[9] which involves addition of a calibrated antiserum to an unknown amount of antigen. From the undissociated serum, more or less the same amount of antigen was recovered as was originally added, which is, of course, the expected result since no precipitates formed in those sera. From the dissociated serum, however, less antigen was recovered than was originally added, showing that the precipitates in serum B had removed antigen from solution.

In an antiserum to a pure antigen it is thus possible to estimate nonprecipitating antibody by first removing precipitating antibody, then dissociating the serum, allowing antibody-antigen complex to separate, and finally adding antigen to the supernate and estimating the amount of precipitate obtained.

For this purpose, it is necessary to remove completely the precipitating antibody from the serum. If precipitating antibody is left in solution, it may be possible for such precipitating antibody to aggregate nonspecifically with other proteins during the dissociation procedure and this may give rise to false increases in the amount of precipitate obtained.

The serum used in the preceding experiment was a serum to a pure antigen. However, if the serum had contained antibodies to trace impurities of the injection antigen, a very large amount of antigen would have been necessary to remove all the precipitating antibody, according to Vaughan and Kabat,[10] and this would necessarily have given rise to conditions of antigen excess.

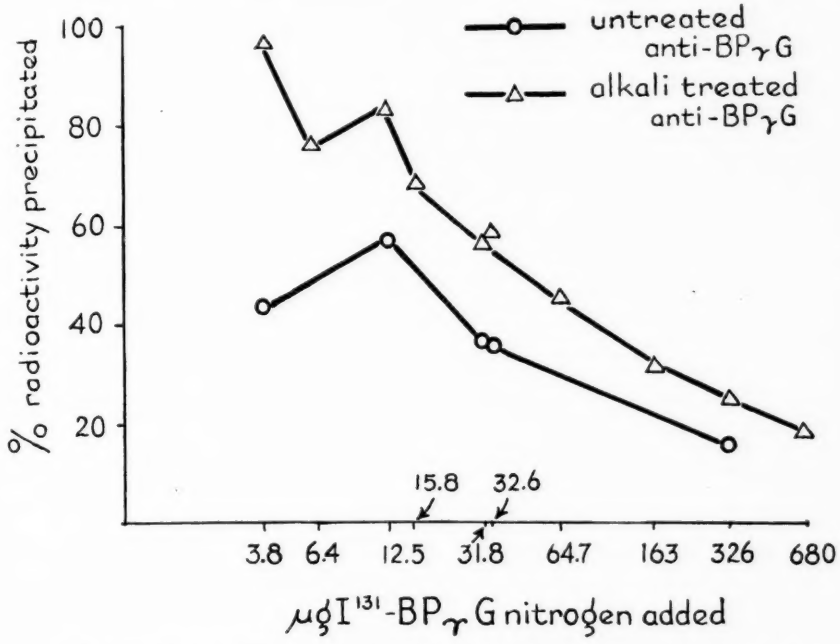


Fig. 4.—Precipitation of radioiodinated bovine plasma gamma globulin by nondissociated (untreated) and dissociated (alkali-treated) anti-BP$_\gamma$G depleted of part of its precipitating antibody. (From Sternberger, Feinberg, and Clarke: *J. Exper. Med.* **103**: 523, 1956.)

Therefore, in a serum containing antibodies to several components of antigen, demonstration of nonprecipitating antibody rests only upon the increased antigen-removal capacity of the serum after dissociation, as compared to this property before dissociation.

Fig. 4 shows the antigen-removal capacity of undissociated and dissociated serum to bovine plasma globulin. This serum reacted with three components of the antigen. It was depleted of the precipitating antibody to one of these

antigen components only. A portion of this serum was dissociated and antibody-antigen complex was allowed to separate. It was then reacted with varying quanities of bovine plasma gamma globulin labeled with radioactive iodine. Undissociated serum was also reacted with bovine plasma gamma globulin. The undissociated serum formed precipitates because of its content of precipitating antibody, and the dissociated serum formed precipitates because of its content of precipitating and possibly some nonprecipitating antibody. About three times as much nitrogen was precipitated from the dissociated serum as from the undissociated serum. Fig. 4 gives the antigen-removal capacities of the dissociated serum and the undissociated serum. More

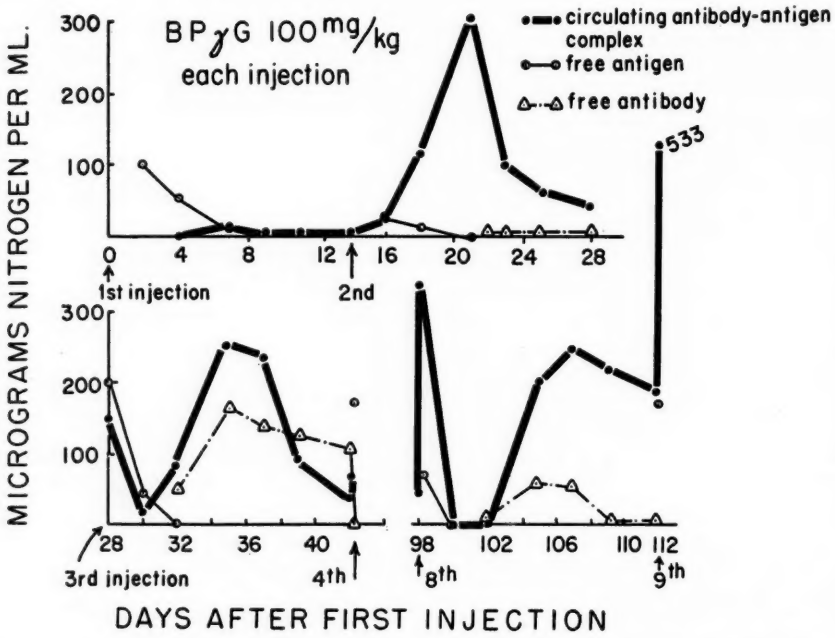


Fig. 5.—Formation of circulating antibody-antigen complex after repeated intravenous injections of 100 mg. crystalline bovine plasma albumin per kilogram of body weight. (From Sternberger.[15])

radioactivity was removed by the dissociated serum, showing that the dissociation uncovered antigen-precipitating activity not apparent in nondissociated serum. Nonspecific precipitation of radioiodine-labeled bovine plasma globulin by an unrelated system of nonprecipitating antibody and antigen did not occur.

With this information on nonprecipitating antibody, we may now return to the significance of antibody-antigen complex and to its relation to antigen persistence. Fig. 5 shows the immune response of a rabbit receiving 100 mg. per kilogram of bovine plasma gamma globulin every fourteen days. After the first injection antigen disappeared by the ninth day; free antibody was not found and antibody-antigen complex appeared in trace amounts between the ninth and the fourteenth days. After the second injection antigen disappeared more rapidly, as expected. Free antibody, though in trace amounts

only, now appeared on the twenty-first day, seven days after reinjection. Antibody-antigen complex rose as early as the second day after reinjection and reached a maxium of 302 meg. nitrogen per milliliter on the twenty-first day, seven days after the second injection. This appearance of antibody-antigen complex earlier and in larger amounts than after the first injection seems to indicate an anamnestic response of the nonprecipitating antibody which enters into formation of the antibody-antigen complex.

After the third and later injections, an additional response was noted. Thus, on the twenty-eighth day, before the third injection, the serum contained 47 meg. nitrogen antibody-antigen complex per milliliter. Immediately after injection, it contained 147 meg. nitrogen per milliliter. Such an immediate rise of antibody-antigen complex is again shown on the ninty-eighth day for the eighth injection, and on the 112th day for the ninth injection. It was often observed in reinjection experiments, but failed to occur if the serum contained a very large amount of precipitating antibody before injection. Apparently, only part of the antigen injected combines with precipitating antibody, while the remainder combines with nonprecipitating antibody and exists in circulation as antibody-antigen complex.

The complex does not persist in circulation for a long time. Two days after injection, it is eliminated from circulation. Both the antibody-antigen complex and the free antigen disappear by that time. Apparently both free antigen and bound antigen are removed by combination with precipitating antibody.

However, usually on the fourth day, antibody-antigen complex reappears. This reappearance of antibody-antigen complex is earlier than its appearance after the first injection, which was on the ninth day, and indicates an anamnestic response of the antibody forming the antibody-antigen complex.

The question of where the antigen comes from arises. There was no antigen in circulation on the second day after reinjection and, therefore, the antigen necessarily must have come from the tissues. What are the nature and significance of this reappearance of antigen? The antigen is first present in the serum. Then on the second day it disappears from the serum, apparently by combination with precipitating antibody, and on the fourth day it reappears, apparently in combination with nonprecipitating antibody. We thought that this might have some significance. Perhaps the antigen is metabolically altered while it is in the tissues.

Dixon and associates[2, 3] have shown that radioiodine-labeled antigen is rapidly eliminated from circulation and that no antigen is found or reappears in the serum by the time precipitating antibody is detected. On the other hand, most theories of antibody formation require the continued presence of antigen in order to explain the molding of antibody. This morning we have heard from Dr. Edsall that after one, two, or three injections of antigen antibody may persist in immunized individuals for a very long time. Since the half-life of antibody is apparently of the order of magnitude of five days, this must imply the continued formation of antibody.

McMaster and associates[11] have shown that antigen persists in the serum of rabbits for as long as four weeks after a single intravenous injection of bovine plasma globulin. They have demonstrated the antigen by injection of the rabbit serum into adrenalectomized mice; the mice were challenged two days later by a strong antiserum to bovine plasma globulin, and an anaphylactic reaction was observed. Either gross anaphylaxis occurred or a sensitive ear vascular response was utilized.

When we obtained sera from rabbits four weeks after injection with bovine plasma globulin, and reacted such serum with precipitating antiserum to bovine globulin, we could, of course, never observe any precipitates, but when we reacted it with nonprecipitating antibody disclosed by dissociation we did obtain precipitates. Apparently there persists an antigen in circulation which reacts with nonprecipitating antibody but does not react with precipitating antibody.

We can now explain why radioiodinated antigen disappears rapidly from the circulation if its fate is followed by radioactivity measurements. Apparently, the antigen originally injected is rapidly degraded, but there persists in circulation a material which is structurally slightly different from the original antigen. The change may be sufficient to involve loss of the iodine label. This material does not react any more with precipitating antibody, but it is capable of reacting with nonprecipitating antibody and it may partake in some reactions of hypersensitivity.

Freund[12] suggested a few years ago that immune globulin initiating in the host may give some protection to antigen against destruction or elimination. Perhaps reaction of antigen with nonprecipitating antibody and its persistence as a soluble antibody-antigen complex comprise this protecting factor. The failure of such modified antigen to react with precipitating antibody may be an important factor, permitting its persistence and preventing elimination by precipitation. We do not know whether the antigen which persists in circulation is a modified material related to the orginal antigen as one of its breakdown products or whether it is a new material formed by the host in the response to the antigen injected.

Whatever its nature is, it seems to have a similar specificity to nonprecipitating antibody as the original antigen, but it does not react with precipitating antibody. The original antigen, on the other hand, reacts specifically with precipitating as well as some nonprecipitating antibody but seems to react with other fractions of nonprecipitating antibody in a manner similar to a cross-reacting antigen. Evidence does not permit anything other than speculation as to whether the formation of this modified antigen is an important stage in the production of antibody. It raises several questions which at present cannot be answered. For example, is such modified antigen a significant constituent of the transfer cells of Chase, or is the formation of soluble antibody-antigen complex a factor in permitting persistence of antigen and continued formation of antibody, as often implied in allergy?

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## Discussion

DR. FEINBERG.—I have been closely in contact with this work for the last year and I feel that, even from the clinical aspect, it has tremendous implications, such as the question of conversion of one antibody to another, the question of persistence of antigen, the question of anamnestic response, the question of the possibility of the antigen being in the tissue and not in circulation, and so forth.

DR. ELLIOT MIDDLETON.—I would like to ask one question, Dr. Sternberger. In the sera which are supposed to contain both the partially degraded antigen and nonprecipitating antibody, was it possible, with those sera, to inhibit the reaction of the precipitating antiserum with added antigen? In other words, if there was a partially degraded antigen capable of inhibiting the reaction present in the serum and not completely bound by the nonprecipitating antibody, could it be shown to inhibit the full precipitating reaction?

DR. STERNBERGER.—We have not done that experiment, and the reason was that degraded antigen does not react with precipitating antibody. In order to do such an inhibition experiment, we would need an antigen with higher avidity for precipitating than for nonprecipitating antibody.

DR. MIDDLETON.—The full antigen has a higher avidity than the partly degraded one?

DR. STERNBERGER.—When the original antigen combines with precipitating antibody, the antigen is removed from solution, while in the combination with nonprecipitating

antibody the complex remains soluble. Therefore, even if the avidity of the original antigen toward both types of antibody were comparable, most of the antigen would be removed from the system by precipitating antibody. It appears that nonprecipitating antibodies are heterogenous in their avidity toward the original antigen, some having lower and others higher avidity than precipitating antibody. There is also a technical difficulty, since in experiments of this kind we have to detect total protein precipitated as well as the original and the degraded antigen incorporated in a precipitate. This would require independent labeling of the original and the degraded antigen.

DR. MERRILL CHASE.—It would be likely, then, would it not, that such complexes would have escaped the attention of Hawn and Janeway[13] when they were studying the kind of serum sickness that arose following the injection into rabbits of bovine plasma globulin and bovine albumin?

DR. STERNBERGER.—Well, it was actually the work of Hawn and Janeway which started us out on these experiments. That is why we used large doses of bovine plasma globulin at first. But I wonder whether they observed something which was not, perhaps, due to the formation of modified antigens or the persistence of modified antigens and their reaction with nonprecipitating antibody.

DR. CHASE.—But one need make, I think, no inferences with regard to a histologic lesion. Certainly, something could have been there and could have been missed when they were trying to equate the day of the histologic event with the day of the appearance of circulating antibody.

DR. STERNBERGER.—Yes, but in a way I think the experiments hold just as much, because wasn't it when the antibody appeared that they found appearances of lesion, that is, when antibody appeared and complement disappeared from circulation?

DR. CHASE.—No, in one of them they found the lesion before the circulation of antibody could be demonstrated. I believe that was with the bovine gamma globulin.

DR. STERNBERGER.—Well, of course, it might have some implication, because we find antibody-antigen complex already at the time when antigen is still present in circulation.

DR. CHASE.—That would probably be it.

DR. STERNBERGER.—Yes. Thank you for drawing my attention to this.

DR. MIDDLETON.—Dr. Sternberger, I did not understand just what your feeling is as to why this nonprecipitating antibody will not coprecipitate in the usual fashion.

DR. STERNBERGER.—Oh, I don't say it never coprecipitates! As a matter of fact, we can do an experiment which, by implication, may help to answer your first question, made earlier in this discussion. We can obtain a precipitate of antibody-antigen complex composed of the injected antigen and the nonprecipitating antibody converted to a precipitating antibody. When we add to this precipitate modified antigen which is radioiodine-labeled we get an exchange reaction of this modified antigen with the precipitate. But we do not necessarily inhibit the precipitate. We obtain a little more precipitate, probably because the original precipitate was made in antibody excess.

DR. JULES FREUND.—What happens to the complement during this time when the antigen and antibody coexist in the serum? A second question, if I may ask another one, is: Can you produce sensitization with this antibody which is nonprecipitating?

DR. STERNBERGER.—We have done complement determinations, Dr. Freund, only in the rabbits which have received large doses of antigen, 1 Gm. per kilogram of bovine plasma globulin, and found complete disappearance of complement, regularly, just the

same as Hawn and Janeway found it. As yet, unfortunately, we have not done the sensitization experiments. I know that we should do them, but we have not had the time.

DR. FREUND.—May I make one remark, that the best tool for research, to bring about interesting results, is the brain of the research worker.

DR. STERNBERGER.—Well, that is why meetings are very important, and I am grateful for any such assistance as we may receive from participants in this seminar and from other people at a meeting. Thank you very much.

MEMBER OF AUDIENCE.—Dr. Sternberger, are these soluble antigen-antibody complexes antigenic in animals of the same species?

DR. STERNBERGER.—Yes, they are.

MEMBER.—If so, what is their specificity?

DR. STERNBERGER.—If we collect serum containing soluble antibody-antigen complex, treat the serum by dissociation, obtain a precipitate, wash this precipitate, and then inject this rabbit antibody-antigen precipitate into guinea pigs along with Freund's complete adjuvants, then we obtain antibodies to the antigen part and to the antibody part of this complex.

DR. MIDDLETON.—Wouldn't you think, from that evidence right there, that there should be determinant groups available for combination with precipitating antibody originally, then?

DR. STERNBERGER.—Yes, there should if the antigen were not modified. Nonprecipitating antibodies may vary in avidity for antigen. Low-avidity nonprecipitating antibody seems to coprecipitate, while high-avidity nonprecipitating antibody seems to be able to "block" antigen and retain it in solution in competition with precipitating antibody. Nearly all the nonprecipitating antibody formed after few intravenous injections of antigen is of the latter type. After immunization with Freund's adjuvants, the coprecipitating variety seems to be predominant.

# NONSPECIFIC FACTORS IN IMMUNITY AND HYPERSENSITIVITY

IRWIN H. LEPOW, PH.D.,* CLEVELAND, OHIO

I SHALL restrict my remarks on nonspecific factors in immunity and hypersensitivity to two immunochemical systems in blood, namely, the complement system and the properdin system. This is done not to negate or minimize the vast importance of cellular factors or of other humoral factors, but rather to focus attention in the very short time available on a group of substances which are under active investigation and which give promise of providing links in the mechanisms of immunity and hypersensitivity.

We have known for some time that a group of factors, collectively known as "complement," is present as a normal constituent of fresh serum; that there are probably four components of complement, which we designate C′1, C′2, C′3, and C′4; and that these factors can enter into antibody-antigen reactions in a variety of ways to give a variety of observable effects. The familiar effects, of course, are the lysis of sensitized erythrocytes and the lysis or killing of gram-negative bacteria which have been sensitized with specific antibody, but other effects are also known.

We know that in the case of soluble antigens, such as egg albumin, for example, or the pneumococcal polysaccharides, the antigen-antibody complex is not grossly altered in the presence of complement, but we can show that complement activity has disappeared by indirect testing, using sensitized erythrocytes as an indicator system. This, of course, is the basis of diagnostic complement fixation tests.

This phenomenon of complement fixation, that is, the disappearance of complement activity following treatment of fresh serum with antigen-antibody aggregates, has been of some interest to us in our laboratory.

It is certainly not a fresh idea that, in addition to its role in protective immunity, complement may also play a role in hypersensitivity. We know that complement activity is depressed in anaphylaxis, serum sickness, and glomerular nephritis, to mention but a few entities, and we know also, as was indicated just a short time ago, that, for the most part, the time of fall of complement activity can be correlated with the appearance of cellular damage. The teleologic argument is that complement is not present merely to be inactivated and that perhaps complement may participate in the chain of reactions leading to cellular injury.

In this connection, we call to mind the work of Bronfenbrenner[1] and of Jobling and associates,[2] and the concept of antigen-antibody reactions liberating anaphylatoxin as a result of activation of a serum protease. We also recall the

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*Assistant Professor of Biochemistry, Western Reserve Medical School.

experiments made by Kulka[3] about a dozen years ago, showing that for the consistent production of smooth muscle contractions in the Schultz-Dale apparatus, a factor resembling complement was required in addition to antibody and antigen.

It has appeared to us that if we could discover the mechanism of complement fixation, we might begin to interpret the meaning of the fall in complement activity which is observed in certain hypersensitivity states and to interpret whether this fall is a cause or an effect phenomenon in terms of the observed injury.

Since time does not permit me to outline some of the experiments that we have done on the mechanism of complement inactivation by antigen-antibody aggregates, as well as by the plasma proteolytic enzyme system with which we have dealt, I shall merely summarize, in a few sentences, the current status of the problem.[4-7]

We have been able to separate the gross phenomenon of complement fixation into two distinct reactions. The first reaction involves antigen-antibody and the first component of complement and has the characteristics of an adsorption. This reaction proceeds instantaneously at 1° C. and independently of pH within the pH range of stability of C′1, the first component; it is insensitive to changes in salt concentration, and it seems to be independent of the presence or absence of divalent cations.

The second reaction involves some product of the reaction between antigen-antibody and C′1, and results in inactivation of the second and fourth components of complement, which we designate C′2 and C′4. This reaction is time dependent; it proceeds much better at 37 degrees than at lower temperatures; it is potentiated by the presence of calcium ions rather specifically; and it shows a marked dependence on pH, with an optimum at about pH 7, and a marked dependent; it proceeds much better at 37° C. than at lower temperatures; reaction, therefore, has characteristics which are suggestive of an enzymatic mechanism.

The gross impression that I would like to leave after this brief discussion is that we are trying to arrive at a mechanism of complement fixation because it may be possible to correlate such basic information with the sequence of events in certain hypersensitivity states. For example, we have drawn the hypothesis that C′1 is a proenzyme which may be activated by antigen-antibody aggregates, and that the active enzyme is responsible for the inactivation of C′2 and C′4 in complement fixation. If such a mechanism were indeed operating, we should like to know the relation, if any, of C′1-activation to the protease activation referred to by earlier workers and the relation, if any, of inactivated C′2 and C′4 to the concept of anaphylatoxin. Perhaps we shall be in a position to discuss these speculations on an experimental basis sometime in the future.

I should like to devote the bulk of my time to a discussion of the properdin system. Properdin has been in the literature now for less than one year. It is a serum protein, a normal constituent of all mammalian sera that have

been tested. In conjunction with complement, or factors which closely resemble components of complement, and in conjunction with magnesium ions, properdin appears to play a rather important role in natural or nonspecific immunity.

It is still too early to give you any idea of what the role of the properdin system in hypersensitivity or allergy might be. This is a problem which we hope will be tackled in the near future. I should like, however, to outline something about the properdin system and what we know about its role in natural immunity.

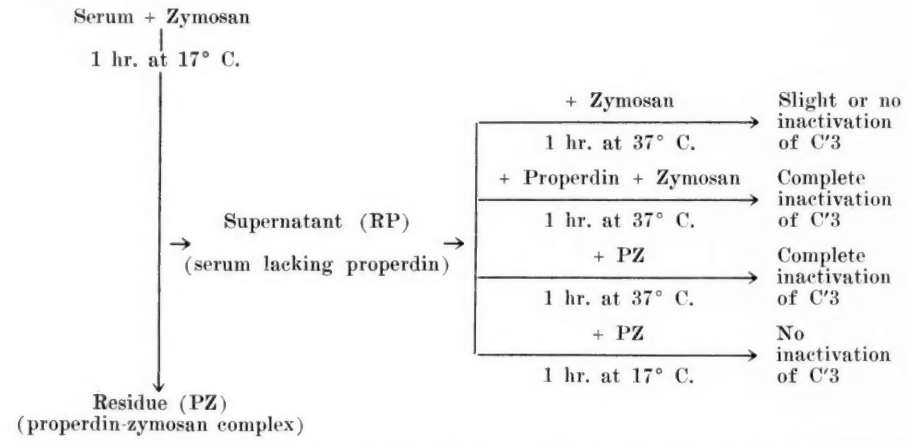


Fig. 1.—The combination of zymosan with a substance (properdin) in human serum that is necessary for the inactivation of C'3 by zymosan at 37° C. (From Pillemer, Blum, Lepow, Ross, Todd, and Wardlaw: *Science* **120**: 279, 1954.)

The history of this problem is rather interesting. Dr. Pillemer was interested, several years ago, in isolating the third component of complement. It was well known that a substance called zymosan, which is essentially an insoluble carbohydrate derived from the yeast cell wall, could rather specifically inactivate the third component of complement. It was assumed, without any investigation of the problem ever having been made, that since zymosan was an insoluble material, it probably acted as an absorbent. This, then, appeared to be a means to isolate the third component. One would merely have to treat serum with zymosan, spin down the insoluble complex, and then elute the third component. However, all attempts to do this were unsuccessful.

In the course of studying the reaction, it became clear that it was not a classical adsorption. For example, this apparently simple inactivation of the third component of complement by zymosan required magnesium ions.[8] It had sharp pH and temperature optima, and it required the presence of other factors, factors which resembled components of complement.[9]

The inactivation of the third component of complement by zymosan is usually accomplished at 37° C. When an experiment was run in which the serum was treated with zymosan at 17° C. and the precipitate was centrifuged off, it was found that no matter how much zymosan one added to the supernatant, one could no longer inactivate the third component of complement at

37° C. Something was removed by the zymosan at 17° C. which, in addition to complement-like factors and magnesium ions, was required for the inactivation of the third component by zymosan.

Fig. 1 shows the type of system which led Pillemer[10] to the discovery of properdin. For reasons which will soon be apparent, the name was taken from the Latin *perdere,* meaning "to destroy." If serum is treated with zymosan at 15° or 17° C. for one hour and then centrifuged, we get a residue which is a complex of zymosan and properdin (PZ). The supernatant (RP), which lacks properdin, contains all the components of complement essentially in their full titers.

If zymosan is added to this supernatant and incubated at 37° C. for one hour, we find little or no inactivation of the third component. However, if the original serum had been treated at 37° C. with zymosan, the third component would have been inactivated.

If zymosan and properdin are added to the supernatant, RP (the isolation of properdin will be discussed in a moment), and incubated at 37° C. for one hour, we do get inactivation of the third component. Hence, properdin is the factor which was removed at 15° to 17° C. and which is required for the reaction with C'3 at 37° C.

Similarly, if we add the PZ complex to the supernatant (RP) the reaction goes at 37° C. and we get complete inactivation of the third component. However, if we add PZ at 15° C. and incubate for one hour, we find no inactivation of the third component.

TABLE I. A COMPARISON OF THE FACTORS AND CONDITIONS REQUIRED FOR THE COMBINATION OF (1) HUMAN PROPERDIN WITH ZYMOSAN AND (2) ANTIBODY WITH ANTIGEN

| HUMAN PROPERDIN WITH ZYMOSAN REQUIRES | ANTIBODY WITH ANTIGEN DOES NOT REQUIRE |
|---|---|
| $Mg^{++}$ ions | $Mg^{++}$ ions |
| Complement | Complement |
| Temp. $>$ 10° C. | Temp. $>$ 10° C. |
| *p*H 6.5 to 8.2 | *p*H 6.5 to 8.2 |
| $\mu = < 0.4$ | $\mu = < 0.4$ |

(From Pillemer, Blum, Lepow, Ross, Todd, and Wardlaw: *Science* **120**: 279, 1954.)

The possibility immediately presents itself that this new substance, properdin, may be nothing more than an antibody to zymosan. We have tabulated a comparison of the requirements for the combination of antigen with antibody and of properdin with zymosan, and it will be noted that the two systems are quite different (Table I). The antigen-antibody system, of course, does not require magnesium ions or any other specific ion and it does not require complement. Combination occurs over a wide range of temperature, pH, and ionic strength. In contrast, the properdin system requires (1) the presence of magnesium ions, (2) factors which resemble components of complement, and (3) a temperature greater than 10° C.; furthermore, it has rather sharp pH and ionic strength optima.

Table II shows further the difference between the antigen-antibody complex and the properdin-zymosan complex in terms of the effects on complement components.

TABLE II. A COMPARISON OF THE EFFECT OF (1) PROPERDIN-ZYMOSAN COMPLEX (PZ) AND (2) ANTIBODY-ANTIGEN AGGREGATES (ANTI-SIII-SIII) ON HUMAN COMPLEMENT COMPONENTS

| HUMAN SERUM AND RP TREATED WITH | EFFECT ON COMPLEMENT COMPONENTS |
|---|---|
| PZ for 2 hr. at 37° C. | Complete loss of C′3; no loss of C′1, C′2, C′4 |
| Anti-SIII-SIII for 2 hr. at 37° C. | Slight loss of C′3; almost complete loss of C′1, C′2, C′4 |
| PZ for 18 hr. at 1° C. | No loss of any of the components |
| Anti-SIII-SIII for 18 hr. at 1° C. | Slight loss of C′3; almost complete loss of C′1, C′2, C′4 |

(From Pillemer, Blum, Lepow, Ross, Todd, and Wardlaw: *Science* **120**: 279, 1954.)

A classical antigen-antibody system, such as rabbit anti-SIII serum and pneumococcus SIII, when treated with fresh serum for two hours at 37° C. or for eighteen hours at 1° C., causes very little change in the titer of the third component. However, almost complete loss of C′1, C′2, and C′4 are observed.

On the other hand, the properdin-zymosan complex, when treated with fresh serum for two hours at 37° C., causes complete loss of C′3 but no loss of C′1, C′2, and C′4. Furthermore, little or no reaction occurs during eighteen hours at 1° C.

A tabulation of some of the properties of human properdin is given in Table III. It is a euglobulin with minimum solubility between pH 4.8 and

TABLE III. PROPERTIES OF PURIFIED HUMAN PROPERDIN

1. Euglobulin with minimum solubility between pH 4.8 and 6.5
2. Sedimentation constant about 27 S
3. Represents not more than 2 to 4 mcg. of protein nitrogen per milliliter of serum, or about 0.03 per cent of the serum proteins
4. Stable to heating at 66° C. for 30 min.
5. Destroyed at 100° C. in 5 min.
6. Not a component of the blood-clotting, plasmin, or hemolytic complement systems
7. Participates in bactericidal, virus-neutralizing, and hemolytic reactions in the presence of complement and $Mg^{++}$

(From Pillemer, Blum, Lepow, Ross, Todd, and Wardlaw: *Science* **120**: 279, 1954.)

6.5. The sedimentation constant in the ultracentrifuge is about 27S. The minimum molecular weight is about 1,000,000, but no measurements of shape have yet been made. It represents only 2 to 4 mcg. of protein nitrogen per milliliter of fresh serum, which is only about 0.03 per cent of the serum protein. In the purified state, it is stable to heating at 66° C. for thirty minutes. In fresh serum, however, it is rapidly destroyed at 56° C. It is not a component of the blood-clotting system. Similarly, it is not a component of the plasmin system or of the hemolytic complement system. As I shall elaborate in more detail, properdin participates in bactericidal, virus-neutralizing, and hemolytic reactions in the presence of complement and magnesium ions. Thus, the importance of properdin goes well beyond its requirement for the inactivation of the third component by zymosan.

A general flowsheet for the preparation of properdin from mammalian serum is shown in Fig. 2. We have prepared properdin from human, bovine, and porcine sera. It probably can be prepared from a great many other mammalian sera as well.

The serum is treated with zymosan at 15° to 17° C. for one hour and centrifuged. The residue, which is the PZ complex, is washed free of occluded serum proteins and is then eluted with a buffer of pH 7.4, at relatively high ionic strength, at 37° C. Under these conditions, the PZ complex dissociates and properdin goes into the soluble phase.

The eluate is then dialyzed against water at 1° C. and properdin precipitates. It can be dissolved in buffer at pH 7.4, and it can be further purified by differential centrifugation. An inactive fraction is taken off at 25,000 g for 3 hours, and the active material is centrifuged down at 75,000 g for twenty hours. A pellet is obtained in the ultracentrifuge, which represents highly purified, but not pure properdin in a yield of 20 to 50 per cent. The purification factor is 3,000-fold compared with the initial serum.

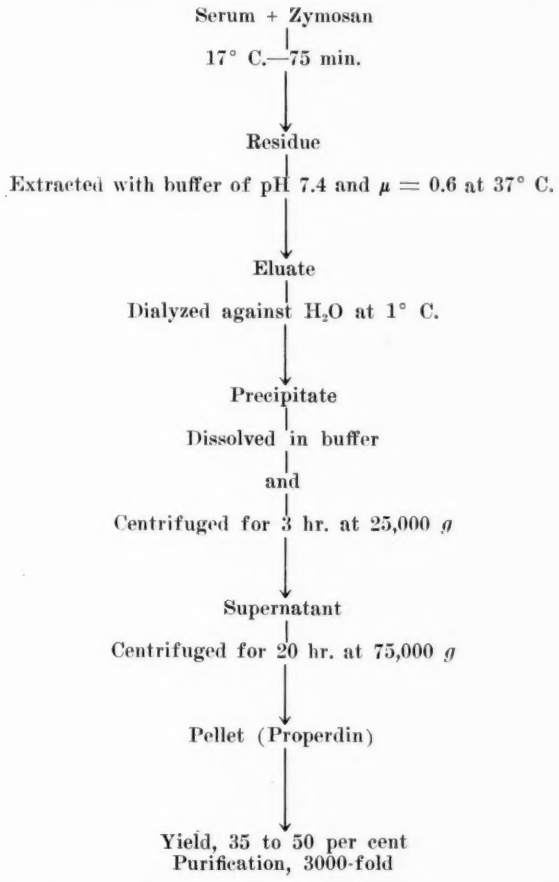


Fig. 2.—Flow diagram for the isolation of human or bovine properdin. (From Pillemer, Blum, Lepow, Ross, Todd, and Wardlaw: *Science* **120**: 279, 1954.)

In Table IV a comparison is shown of the various activities of fresh human serum and the activities of RP, that is, serum which has been made deficient in properdin by treatment with zymosan at 17° C. It will be noted

TABLE IV. COMPARISON OF THE FACTORS AND ACTIVITIES OF UNTREATED HUMAN SERUM AND PROPERDIN-FREE SERUM (RP)

| FACTOR OR ACTIVITY | IN UNTREATED SERUM | IN PROPERDIN-FREE SERUM (RP) |
|---|---|---|
| Hemolytic activity for sensitized sheep cells | Present | Present |
| Individual complement components | Present | Present |
| Susceptibility to complement fixation | Present | Present |
| Plasminogen | Present | Present |
| Plasmin inhibitors | Present | Present |
| Clotting factors | Present | Present |
| Hemagglutinin | Present | Present |
| Susceptibility of C'3 to inactivation by zymosan | Present | Absent |
| Heat-labile bactericidal activity | Present | Absent |
| Heat-labile virus-neutralizing activity | Present | Absent |
| Hemolytic activity for unsensitized cells | Present | Absent |

(From Pillemer, Blum, Lepow, Ross, Todd, and Wardlaw: *Science* **120**: 279, 1954.)

that in both the fresh untreated serum and the RP, hemolytic complement activity and the individual complement components are present. Similarly, both the untreated serum and the RP are equally susceptible to complement fixation by antigen-antibody aggregates, and both contain equal amounts of plasminogen, plasmin inhibitors, clotting factors, and hemagglutinin.

However, there are important differences also. The susceptibility of C'3 to inactivation by zymosan is present in untreated serum but absent in RP. This, of course, was the observation which led to the discovery of properdin.

I should like to discuss now other activities of the properdin system which were mentioned but not elaborated upon earlier.

Ginsberg and Horsfall,[11] some years ago, reported that fresh serum contained a heat-labile, virus-neutralizing activity which required complement and a divalent cation for its action. In the course of the present work, Ginsberg tested untreated serum and RP against Newcastle disease virus. He found that, whereas this heat-labile, virus-neutralizing activity was present in the original serum, it was absent in RP. Furthermore, the heat-labile, virus-neutralizing activity of RP could be reconstituted by the addition of purified properdin.

Similarly, a heat-labile, nonspecific antibacterial activity directed toward a wide spectrum of organisms is known to be present in fresh serum. Direct in vitro experiments have been carried out in our laboratory by Dr. Wardlaw and have shown that many gram-negative enteric organisms are killed by fresh serum and are not killed by RP, and that if one adds purified properdin to RP bactericidal activity is restored.

Both the bactericidal and virucidal systems require $Mg^{++}$ and factors resembling components of complement for their activity. Susceptible bacteria or viruses cannot be killed by fresh serum from which a component of complement has been removed. Similarly, if we add properdin back to RP, which reconstitutes the system, and then inactivate a component of complement or eliminate magnesium ions, bactericidal and virucidal activities are absent.

Another line of evidence for the role of properdin in antibacterial activity is provided by experiments with radiation illness. It has been found possible to protect rats and mice against the enteric invasion following irradiation by

the administration of properdin at appropriate times. The time interval is rather critical but, under properly controlled conditions, if irradiated animals are given properdin a significant percentage of animals which would have died will survive.

There are also experiments by Rowley[12] in England showing that if zymosan or cell walls derived from certain *E. coli* strains are injected into mice one-half hour before challenging with *E. coli,* an avirulent strain of *E. coli* will behave as a virulent strain. On the other hand, if these mice are injected with cell walls forty-eight hours before challenging with *E. coli,* the animals are protected against even the virulent strain. This type of experiment has been performed in our laboratory and we can correlate both Rowley's results and our own with the properdin titer of serum at any given time. The properdin titer drops precipitously during the half-hour following injection of cell walls, and mice become highly susceptible to infection by normally avirulent *E. coli.* However, this initial drop in properdin is not only rapidly compensated but is overcompensated and we may find at forty-eight hours a properdin titer two to four times the normal level. Such animals are resistant to the normally virulent strain of *E. coli.*

There are also experiments with *Klebsiella* along the same lines, in which we can predict with some accuracy how many animals are going to survive and how many animals are going to die, on the basis of the properdin titer at the time of challenge.

With respect to the hemolytic activity for unsensitized erythrocytes, we are dealing here with abnormal cells. Erythrocytes of patients with paroxysmal nocturnal hemoglobinuria are lysed in a system which does not require specific antibody, but which does require magnesium ions and components of complement. It has been shown that properdin is also required, so that in untreated serum the activity for lysing PNH cells is present, whereas in RP it is absent. Once again, addition of purified properdin to RP restores activity.

Very recently, it has been shown also that erythrocytes which have been treated with tannic acid are lysed through the properdin system and that RP is hemolytically inactive, whereas untreated serum is active. Again, the same factors are required—properdin, magnesium ions, and components of complement.

I wish that I had some information to present on the role, if any, that properdin plays in allergy and hypersensitivity. Certainly, this will be a fascinating area of investigation.

I should like to leave with just one remark on the diversity of reactions in which complement is known or suspected to participate. Complement plays a role in protective immunity in the classical antigen-antibody system; it may be involved in some way in the backfiring of protective immunity which we call hypersensitivity; it is a requirement in a natural immune system, the properdin system; and through the properdin system, it may also play an additional role in hypersensitivity and allergy.

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## Discussion

DR. GEOFFREY EDSALL.—I have one question. I did not catch any mention of the properdin levels in acute infections. Did you feel that was too complicated or too uncertain to bring up at the moment?

DR. LEPOW.—In animal work, we can correlate susceptibility to infection and the eventual course of the infection with the properdin level. We have not done too much work in following human infections as yet, partly because of the complications of the assay system, particularly with reference to human serum. However, this is certainly an area which will be followed in the future.

MEMBER OF AUDIENCE.—You mentioned that properdin was a euglobulin fraction. I wonder where it falls in the fractionation pattern; is it alpha, beta, gamma, or more than one?

DR. LEPOW.—It would fall in the beta-globulin fraction. It is present in Fraction III of the Cohn fractionation schemes.

DR. TALMADGE.—I should like to ask if properdin may be considered a type or a form of natural antibody against the bacteria normally present in the enteric tract. Connected with this question, is the requirement of complement for the absorption of properdin by zymosan also true for the absorption of properdin by the bacteria and the red cells?

DR. LEPOW.—In reply to the second question, definitely. All these reactions which I have outlined—the virus-neutralizing activity, the heat-labile, antibacterial activity, the reaction against abnormal erythrocytes, and the reaction of the third component of complement with zymosan—require not only the presence of properdin but also magnesium ions and factors which resemble the components of complement.

As for the first question, we would like to look upon properdin at this stage of the investigation as a nonspecific agent directed, for the most part, toward gram-negative bacteria.

Several interesting things have come out of Dr. Wardlaw's work. Not all gram-negative bacteria, even of very related species and types, are susceptible to the properdin system. We are not sure of the reason for this. One thing which immediately enters into consideration is the possibility of a competition between the immune system and the properdin system. If the test serum contained specific antibodies, would not these, perhaps, compete with the properdin system and obscure its effects? This is the type of problem that Dr. Wardlaw is contending with now.

DR. JULES FREUND.—I have two short questions. One is: Does properdin have any effect on phagocytosis? Some years ago the prognosis was exceedingly poor. That would suggest that perhaps properdin has some effect on phagocytosis. The other question is: How about age and properdin? Newborn animals are very susceptible, for instance, to polio, in the case of mice and hamsters. Does the newborn animal have properdin?

DR. LEPOW.—Some preliminary experiments were performed by Dr. Wedgwood in our laboratory in an attempt to determine the role of properdin in phagocytosis. His experiments, as far as he carried them, were negative.

As for the newborn, this again is an area to be investigated. In the few infants that have been measured, a normal properdin titer was found. A few sera with agammaglobulinemia have been tested. This is very preliminary again, but it appears that in congenital agammaglobulinemia, the properdin level may be low, whereas in acquired agammaglobulinemia, the properdin level remains within normal limits.

# REAGINIC AND BLOCKING ANTIBODIES

WILLIAM B. SHERMAN, M.D.,* NEW YORK, N. Y.

AT THIS point in the symposium, you may be aware of a sudden change in the nature of the reports, due to some obvious factors. So far, the discussion of antibodies has dealt with normal animals or normal persons subjected to some specific experimental procedure, usually with a highly purified antigen, and the observation of the results from it.

I would imagine that one of the worst things that could happen in an immunologic laboratory would be for all the cages to be opened during the night, and the animals become mixed up, so that the immunologist did not know what treatment any of them had received. That would be exactly the situation with which we deal in clinical allergy all the time.

Our difficulties are increased by the fact that, of all the many substances with which we test and treat patients, I am unable to find one which qualifies as a pure antigen. Finally, in studying the antibody reactions, we are forced to depend almost entirely upon very unsatisfactory biologic indicators of antigen-antibody reaction.

The term *atopic reagin* was applied to the active principle of allergic sera by Coca and Grove[1] because of doubt as to whether it could be considered a true antibody. The reagin was believe to be limited to the human species and to result largely from hereditary influences. It was not even clear at that time whether or not contact with the allergen was important in its development.

With the passage of time and the introduction of new facts, most of these concepts have been modified. In naturally occurring allergy, the hereditary factor is still considered important by most of us, but the development of specific sensitization is now generally believed to result from contact with the antigen. Particularly in regard to geographically distributed antigens, such as pollens, it is generally agreed that persons are sensitive only to antigens with which they may have had previous contact. In the case of foods and household substances, it is often impossible to prove contact or lack of contact. With intense contact with certain antigens, such as horse serum and *Ascaris*, it was soon found that the influence of contact in the production of reagins was so great that they seemed to be developed regardless of the hereditary background. Evidence of similar reaginic substances has since been found in the serum of other species, such as spontaneously sensitized dogs and cattle and experimentally sensitized guinea pigs and rabbits, so they are no longer considered limited to man.

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*Attending Physician, Medicine and Allergy, Institute of Allergy, Roosevelt Hospital; Assistant Attenting Physician, Medicine, and Chief, Allergy Clinic, Presbyterian Hospital, New York City.

I believe that the reagin has come to be considered a type of antibody, more by an extension of the limits of the term *antibody* than by any more typical features that have been observed as time went on. Whether all the antibodies capable of passively sensitizing human skin should be considered reagins is a matter of opinion. For that reason, I prefer to use the simple descriptive term *skin-sensitizing antibody*, rather than *reagin* or *reaginic antibody*.

As you all know, the first evidence of antibodies in human allergy was offered by Ramirez[2] in 1919, when he reported the passive transfer of horse asthma by a transfusion from a patient suffering from horse asthma. This same phenomenon of transfer of sensitivity by transfusion has been more intensively studied by Loveless[3] and others, and it has been shown definitely that when blood from a hay fever patient is transferred into a normal person, the active principle disappears from the blood stream in a relatively few hours, but the skin and eye test become positive and remain so for many weeks, suggesting that the antibody has a marked affinity for these tissues and that its presence in these tissues causes their reaction to the antigen.

The more familiar phenomenon of local passive transfer was described in 1921 by Prausnitz and Küstner,[4] who showed that if serum from an allergic patient is injected intracutaneously into a normal skin, a sharply defined local sensitization was produced. This phenomenon is generally demonstrable with the sera of patients showing well-marked immediate-type urticarial skin reactions, and is the basic method of demonstrating and studying the skin-sensitizing antibody. The only way we can attempt to determine the amount of such antibody is by testing progressive dilutions of the serum and noting the end point at which reaction no longer occurs. Since this depends on the biologic reactivity of the skin of the test subject, which is quite variable, the method lends itself more to comparisons than to absolute measurements.

Concerning the mechanism of the reaction between antigen-antibody, very little can be said except that the more dilute the serum, the higher the concentration of antigen required to produce a detectable reaction.

Fig. 1 represents the results of studies on five test subjects, with a single highly potent anti-castor bean serum. The dotted line and the dots (in which there is a great deal of overlapping) represent the actual observations on five different test subjects, and I have rather arbitrarily drawn what seems to be a reasonable representation of the mean. It is fairly close to a straight line at first and then, as the end point of the serum is approached, all the lines go up off the edge of the graph and thus the mean curves up.

It is equally important to point out that in these five test subjects sensitized with one serum, we find that in one case the end point was 1:100; in two cases it was 1:1,000; and in two cases it was 1:10,000. This sort of variability in different individuals is typical of this phenomenon, so that comparison of two sera on the same subject are more significant than absolute titers.

We believe that the antigen-antibody reaction causes a release of histamine and that the released histamine causes the actual physiologic reaction in the skin. This, obviously, introduces a number of variable factors, which are not the same in every test subject.

The reaction between the antigen and the antibody uses up a certain amount of antibody. After a sufficiently strong reaction, the site is exhausted or neutralized. This is apparent if the same site is tested on successive days with strong concentrations of the same antigen. However, this is not a very satisfactory quantitative procedure because of the difficulty of being sure that the antigen injected in the tests reaches all parts of the sensitized site.

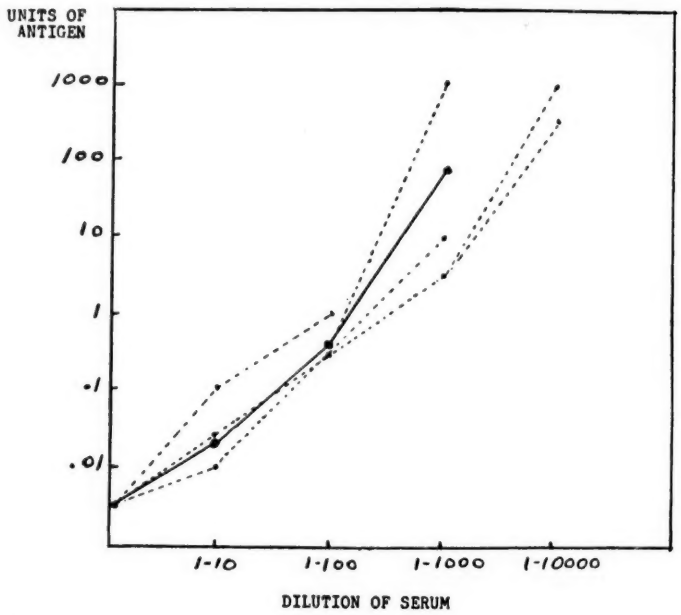


Fig. 1.—Passive transfer tests on five normal subjects with the same anti-castor bean serum. The points represent the lowest concentration of antigen producing a positive reaction.

On the other hand, if a really large excess of antigen is injected into a distant part of the body, or if the test subject eats the food to which the serum is highly reactive,[5] one reaction may occur which completely neutralizes the antibody.

If the sensitizing antibody is mixed with varying amounts of the antigen before it is injected into the skin, subsequent testing with an excess of the antigen shows a rather sharply defined end point or neutralization point (Table I). Disregarding for the moment the dilution tests at the top of the table, it is apparent that in this particular ragweed-sensitive patient the neutralization point of the serum before treatment was 100 units; the second serum, 1,000 units; and the third sample of serum, somewhere between 300 and 1,000 units. Thus, we can measure, in a roughly quantitative way, the amount of antigen required to neutralize the antibody of a particular sample of serum.

As to the action of this component of the serum in the patient himself, we believe, from the transfusion experiments and from the fact that there is at least some correlation between the direct skin test and the content of skin-sensitizing antibody in the serum, that the same antibody which is present

TABLE I. DILUTION AND NEUTRALIZATION TESTS ON SERA OF RAGWEED-SENSITIVE PATIENT AT VARIOUS STAGES OF INJECTION TREATMENT

| | SERUM 1 BEFORE TREATMENT | SERUM 2 AFTER 2 MONTHS | SERUM 3 AFTER 12 MONTHS |
|---|---|---|---|
| DILUTION OF SERUM | DILUTION TESTS | | |
| 1-100 | ++ | ++ | + |
| 1-300 | + | + | + |
| 1-1,000 | 0 | + | 0 |
| 1-3,000 | 0 | 0 | 0 |
| UNITS OF ANTIGEN | NEUTRALIZATION TESTS | | |
| 30 units | ++ | ++ | ++ |
| 100 units | ± | ++ | ++ |
| 300 units | 0 | + | + |
| 1,000 units | 0 | 0 | ± |
| 3,000 units | | 0 | 0 |
| Saline control | +++ | +++ | +++ |

in the serum occurs in the skin and mucosa of the allergic person and is responsible for the occurrence of clinical symptoms or skin tests. Bradel[6] has shown that fluid expressed from nasal polyps of allergic patients contains sensitizing antibodies in concentrations as high as 40 times that present in the serum of the same patient. On the other hand, attempts to recover skin-sensitizing antibody from skin and other tissues than nasal polyps have generally failed.

The human serum capable of passively sensitizing skin does not contain precipitins and does not produce passive anaphylaxis in the guinea pig. Evidence as to whether this skin-sensitizing antibody coprecipitates with antigen and a precipitating serum is confused and inconclusive. Complement fixation with such serum has been reported by Gyorgy, Moro, and Witebsky[7] and, more recently, by Cavelti[8] but the amounts of complement fixed have been too small for measurement and the reaction, in general, is not sufficiently constant for practical use.

It formerly was said that skin-sensitizing antibody was not demonstrable outside the body. In view of the work of Katz and Cohen,[9] this statement obviously must be modified to say that it is not demonstrable in the absence of living cells, for Katz and Cohen showed that the addition of specific antigen to human allergic blood causes a release of histamine from the blood cells into the plasma. This phenomenon has also been studied by Noah and Brand.[11] Spain and his colleagues[10] performed similar experiments, using human allergic serum with rabbit blood as a source of bound histamine.

One of the best-known characteristics of the skin-sensitizing antibody is its thermolability. Activity of serum is destroyed by heating at 56° C. for one-half to four hours and is not restored by the addition of complement. This is of great importance in separating the skin-sensitizing and blocking antibodies; otherwise, I am not sure how characteristic a feature it is.

Eight years ago at the meeting of this Academy, we reported a comparison of the effects of heating for four hours at 56° C. on human and rabbit anti-horse sera which contained both skin-sensitizing antibodies and precipitins.

After heating, the human serum lost both its skin-sensitizing activity and its precipitin activity as measured by the antigen-dilution method. The rabbit serum showed no change in either of these activities. Thermolability, therefore, appeared to be a species characteristic rather than a unique property of the skin-sensitizing antibody or reagin.

I have already mentioned the skin-sensitizing antibody's rather marked affinity for skin and other tissues. In local passive transfer, antibody injected intracutaneously will remain fixed for a period of several weeks without spreading more than 1 cm. in any direction, as anyone who attempts to do these experiments will find out. If he does not mark the site of the sensitizing injection very carefully, he can miss it completely when he makes the test. Unlike most human antibodies and unlike the anaphylactic antibody of guinea pigs, the skin-sensitizing antibody does not pass through the placenta from the maternal to the fetal circulation. This was shown by Bell and Erickson.[13] In later experiments at Roosevelt Hospital,[14] we also found that the placental barrier effectively withheld the skin-sensitizing antibody in forty-seven out of fifty cases, while hemagglutinins, typhoid agglutinins, and diphtheria antitoxin passed through freely. The three exceptions were apparently due to defects in the placenta. In the vast majority of cases, the skin-sensitizing antibody did not, to any extent, pass through from the maternal to the fetal circulation. It was natural to try to relate this impermeability of the placenta to the well-recognized affinity of the skin-sensitizing antibody for tissues, but actually it was impossible to recover any of the skin-sensitizing antibody from the placental tissue.

Concerning the chemical nature of the skin-sensitizing antibody, there is very little to tell. By the older methods of salting out proteins, it was found to occur in the pseudoglobulin. Vaughan and co-workers[15] applied Cohn's method of fractionation with alcohol and found that, while skin-sensitizing activity was not entirely limited to any one fraction, it occured chiefly in Fraction IIId, which consists mostly of beta globulin.

Numerous attempts have been made to identify the skin-sensitizing antibody with one of the main protein components by various methods of electrophoresis. Most workers have found that activity is not limited to any single component, but in various cases it has been found to be present in alpha$_2$, beta, or gamma globulin, depending on the particular procedures used.[16] It is apparent that the skin-sensitizing antibody differs in this respect from most other human antibodies, which occur only in the gamma globulin.

I have already mentioned that it is now generally believed that the natural development of skin-sensitizing antibodies depends upon exposure of the susceptible individual to antigen. Further exposure to the antigen in the injection treatment of hay fever usually produces a rise in the antibody titer during the first two or three months. The changes, in most cases, are from threefold to tenfold, as shown in Table I.

In general, the injection of pollen antigens into nonallergic persons does not produce skin-sensitizing antibody. However, such antibodies are readily produced by the injection of heterologous antisera and *Ascaris* antigen. Kuhns and Pappenheimer[17] have shown that the injection of diphtheria toxoid may produce a skin-sensitizing type of diphtheria antitoxin, particularly in patients with a hereditary tendency toward allergic disease.

Antibodies capable of sensitizing human skin in the same manner as human allergic antibodies may be produced in rabbits and guinea pigs by injections of pollen extract, horse serum, and egg albumin and various other antigens.

The skin-sensitizing activity of the guinea pig antisera is not correlated with the titer of anaphylactic antibody. Table II shows a number of guinea pig antiovalbumin sera, of which the anaphylactic titer in sensitizing doses per milliliter was very carefully determined by Dr. Coulson in the Department of Agriculture. These titers varied from 40 to 400. Five of these nine sera sensitized human skin very well. The serum showing a large titer of anaphylactic antibody did not, and it is obvious that there is no quantitative relationship between the anaphylactic antibody titer and the skin-sensitizing titer.[18]

TABLE II. SKIN-SENSITIZING AND ANAPHYLACTIC ACTIVITY OF SERA OF GUINEA PIGS SURVIVING CHALLENGING DOSES OF OVALBUMIN

| GUINEA PIG NO. | SENSITIZING DOSE,* MG. | INTERVAL BEFORE CHALLENGE | CHALLENGING DOSE,† MG. | INTERVAL BEFORE BLEEDING, DAYS | ANAPHYLACTIC TITRE | SKIN SENSITIZING TITRE |
|---|---|---|---|---|---|---|
| 160 | .02 | 6 wk. | .077 | 6 | 40 | 1-100 |
| 195 | .02 | 6 wk. | .260 | 6 | 100 | 0 |
| 180 | .3 | 6 wk. | .173 | 11 | 133 | 0 |
| 181 | .3 | 6 wk. | .115 | 11 | 100 | 1-10 |
| 706 | .3 | 8 wk. | .39 | 13 | 130 | 1-10 |
| | s. c.‡ | 18 days | 1.0 | 11 | 200 | 0 |
| 707 | .3 | 8 days | .59 | 13 | 400 | 0 |
| 711 | .3 | 8 days | .26 | 13 | 200 | 1-100 |
| | s. c. | 18 days | 1.0 | 11 | 130 | 1-10 |

*Alum precipitated ovalbumin subcut.
†Intrav., in saline sol.
‡s. c. = second challenge.
(From Sherman and Coulson: *Proc. Soc. Exper. Biol. & Med.* 77: 245, 1951.)

The same is true of precipitin titers in rabbits sensitized with egg albumin. Table III shows a series of rabbits sensitized with alum-precipitated egg albumin and a series of rabbits sensitized with soluble egg albumin. There was not much difference in the range of precipitin content of the two groups, but all those treated with precipitated antigen sensitized human skin; none of those treated with soluble antigen did so. Without going into the possible relationship to the way in which they are sensitized, it is apparent that there is no quantitative relationship between the precipitin content and the sensitization of human skin.[19]

From two rabbit antiovalbumin sera, both of which sensitized human skin, the precipitin was quantitatively removed by small stepwise additions of antigen. Removing the precipitating antibody in this manner did not affect the sensitizing power of the antisera for human skin.

TABLE III. ANALYTICAL AND IMMUNOLOGICAL DATA ON RABBIT ANTISERA

| SERUM NO. | PRECIPITIN N (MG. PER ML.) | VALUE OF R CALCULATED | REACTIVITY ON PASSIVE TRANSFER TO HUMAN SERUM DILUTED: UNDILUTED | 1:10 | 1:100 |
|---|---|---|---|---|---|
| **SERA OF RABBITS IMMUNIZED WITH ALUM-PRECIPITATED ANTIGEN** | | | | | |
| 9 | 0.258 | 7.5 | +++ | ++ | ± |
| 10 | 0.381 | 6.1 | ++ | + | − |
| 11 | 0.358 | 9.0 | + | + | − |
| 12 | 0.595 | 9.3 | + | + | − |
| 13 | 0.531 | 10.2 | + | + | − |
| 14 | 0.604 | 10.0 | ++ | + | − |
| 15 | 0.338 | 9.0 | +++ | +++ | +++ |
| 16* | 0.553 | 8.9 | + | − | − |
| 130 | 0.170 | 8.4 | | ++ | +± |
| 132 | 0.047 | 7.5 | | +++ | ++ |
| 135* | 0.071 | 10.0 | | ++ | ++ |
| 164* | 0.102 | 10.6 | +± | ± | ± |
| 165* | 0.087 | 7.5 | ++ | + | + |
| 191 | 0.083 | 9.1 | + | | |
| **SERA OF RABBITS IMMUNIZED WITH DISSOLVED ANTIGEN** | | | | | |
| 1* | 0.088 | 6.7 | − | | |
| 3 | 0.420 | 7.4 | − | | |
| 4 | 0.446 | 8.8 | − | | |
| 5 | 0.193 | 6.2 | − | | |
| 8 | 0.359 | 8.4 | ± | | |
| 2 | 0.05† | | − | | |
| 6 | 0.05† | | − | | |
| 154 | 0.05† | | − | | |
| 155 | 0.05† | | − | | |

*Analysis at two antigen levels only.
†No analysis performed on these sera; values estimated from qualitative sera.
(From Sherman, Menzel, and Seebohm: *J. Exper. Med.* 92: 191, 1950.)

As many of you know, Vaughan and Kabat[20] have pointed out that there is some doubt as to whether this crystalline ovalbumin is a pure enough antigen for this type of experiment and that this may not be the reaction of the pure antigen, but of a number of trace impurities. In any case, it appears that the precipitin does not carry this skin-sensitizing factor.

While the skin-sensitizing antibodies artificially induced by injections of antigens into animals are highly specific, the specificity of naturally occurring skin-sensitizing antibodies is rather vague. Perhaps this is to be expected, as they are formed as the result of casual contact with many different antigens. In passive transfer, it is common to find sera reacting with many different pollens. Do they possess a multitude of different skin-sensitizing antibodies, or does one antibody react with many different antigens? The answer is not easy.

Cross-neutralization experiments with the sera of patients with multiple allergies show many cross reactions to unrelated antigens. For example, in the case of a patient who was clinically very sensitive to dog, but also gave good passive transfer reactions to horse, the addition of dog epithelium extract neutralized the reaction both to dog and horse, whereas an attempt to neutralize with horse epithelium did not affect the reaction to either one.

TABLE IV. A COMPOSITE OF PASSIVE TRANSFER STUDIES SHOWING CROSS NEUTRALIZATION REACTIONS WITH ANTETREATMENT SERUM (WOL.)*

| SERUM MIXED WITH | SERUM TESTED WITH | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | L. RAG. | SORREL | PLANT. | COCKLE. | OAK | HICK. | BIRCH | POPLAR | ASH | TIM. |
| Low ragweed | 0 | + | + | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Sorrel | +++ | 0 | ± | + | ± | 0 | 0 | 0 | 0 | 0 |
| Plantain | +++ | ++ | ++ | ++ | ++ | ± | ± | 0 | ± | 0 |
| Cocklebur | ++ | ++ | ++ | 0 | ++ | + | + | ± | 0 | 0 |
| Oak | +++ | ++ | ++ | + | ± | + | 0 | + | 0 | 0 |
| Hickory | +++ | ++ | +++ | + | ++ | ± | 0 | ± | ± | 0 |
| Birch | +++ | ++ | +++ | ++ | +++ | ++ | + | + | ++ | + |
| Poplar | +++ | ++ | +++ | ++ | ++ | ++ | ++ | + | ++ | + |
| Ash | +++ | ++ | +++ | ++ | ++ | + | + | + | + | + |
| Timothy | +++ | ++ | +++ | ++ | +++ | ++ | + | ± | ++ | ± |
| Control | ++++ | ++++ | ++++ | +++ | +++ | ++ | ++ | + | ++ | + |
| Dilution titer | ±3000 | ±3000 | +1000 | +1000 | ±1000 | ±1000 | ±1000 | ±300 | +100 | +100 |

*This table is arranged in the order of the dilution titer; that is, the reactivity as determined previously by passive transfer tests with the same allergens in serum sites of various dilutions.

This serum diluted 3,000 times still gave slight reactions with extracts of low ragweed and sorrel, whereas with ash and timothy extracts no reaction was obtained after 100 fold dilutions.

(From Cooke: *J. Allergy* **15**: 212, 1944.)

In the reverse situation, in which the patient was clinically very sensitive to horse but gave a moderate passive transfer reaction to dog, attempts to neutralize with dog produced little or no effect, whereas the horse antigen neutralized the reactions to both dog and horse.

In a third case in which the patient was equally sensitive to both dog and horse, each antigen neutralized to itself and did not neutralize to the other.[21]

To show the extent to which cross-neutralization may occur, Table IV shows a study of the serum of a patient sensitive to ten different pollens, which are arranged down and across, roughly in the order of the reactivity to each one. The serum was neutralized with each separate antigen, and then sites were tested with each one. There was a tremendous number of cross reactions and, in general, all those giving strong reactions tended to neutralize to the ones giving weaker reactions.[22]

This reaches a point where it is really very hard to tell whether or not this represents a truly specific immunologic phenomenon.[23] However, there is one other approach to the problem. This has to do with the cases of patients whose serum reacts to two unrelated pollens, but in which we treat the patient

TABLE V. DILUTION TESTS ON WOL. SERUM BEFORE (A) AND AFTER (P) INJECTIONS OF RAGWEED POLLEN EXTRACT

| DILUTIONS | REACTION FROM TEST WITH | |
|---|---|---|
| | LOW RAGWEED 1,000 | PLANTAIN 1,000 |
| | *Serum A (6-13-39)* | |
| 1-500 | ++ | + |
| 1-1000 | ± | 0 |
| 1-2000 | 0 | 0 |
| 1-4000 | 0 | 0 |
| | *Serum P (8-23-39)* | |
| 1-500 | +++ | ++ |
| 1-1000 | ++ | + |
| 1-2000 | + | ± |
| 1-4000 | ± | 0 |

(From Sherman: *J. Immunol.* **40**: 289, 1941.)

with only one of these pollens. Not infrequently, as shown in Table V, treatment with only one of the two pollens produces a roughly parallel increase in the amount of skin-sensitizing antibody to both pollens. The serum of this patient, before treatment with ragweed, reacted in a dilution of 1:1,000 and, after treatment, reacted to ragweed 1:4,000. The patient did not receive treatment with plantain, but the titer for plantain went from 1:500 to 1:2,000, at least suggesting that possibly the same antibody may have reacted with both pollen antigens.[24]

When the patient with hay fever is treated with injections of antigen, the variations in the amount of skin-sensitizing antibody as determined by the dilution test and the neutralization point do not run parallel (Table I). Generally, at the early months of treatment, there is an increase in the amount of skin-sensitizing antibody by the dilution test, but the changes in the neutralization test are usually far out of proportion to those in the titer by the dilution test. Furthermore, with longer treatment, the titer in the dilution test often falls back to its original level, whereas the titer in the neutralization test continues to rise or stays at the same level.

TABLE VI. INHIBITION OF NEUTRALIZATION BY HEATED SERUM OF A TREATED HAY FEVER PATIENT

| SITE | SITES PREPARED WITH MIXTURE OF | REACTION ON RETEST WITH LR 1,000 |
|---|---|---|
| 1 | Nam. A serum (heated) + Hit. A serum + LR 25 | + |
| 2 | Nam. A serum (heated) + Hit. A serum + LR 50 | + |
| 3 | Nam. A serum (heated) + Hit. A serum + LR 100 | 0 |
| 4 | Nam. A serum (heated) + Hit. A serum + LR 200 | 0 |
| 5 | Nam. A serum (heated) + Hit. A serum + Saline | ++ |
| 6 | Nam. P serum (heated) + Hit. A serum + LR 25 | ++ |
| 7 | Nam. P serum (heated) + Hit. A serum + LR 50 | ++ |
| 8 | Nam. P serum (heated) + Hit. A serum + LR 100 | + |
| 9 | Nam. P serum (heated) + Hit. A serum + LR 200 | ± |
| 10 | Nam. P serum (heated) + Hit. A serum + Saline | ++ |
| 11 | Normal serum (heated) + Hit. A serum + LR 25 | + |
| 12 | Normal serum (heated) + Hit. A serum + LR 50 | + |
| 13 | Normal serum (heated) + Hit. A serum + LR 100 | 0 |
| 14 | Normal serum (heated) + Hit. A serum + LR 200 | 0 |
| 15 | Normal serum (heated) + Hit. A serum + Saline | ++ |

A serum = before treatment.
P serum = after treatment.
LR = low ragweed pollen extract.
(From Sherman: *J. Immunol.* **40**: 289, 1941.)

Twenty years ago, at a meeting of this Academy, Cooke, Barnard, Hebald, and Stull[25] presented evidence that this difference between the changes in the dilution and neutralization tests, as a result of treatment, involved the development of a blocking or inhibiting type of antibody, distinct from the skin-sensitizing antibody and competing with it for the same antigen.

Study of the blocking antibody was greatly facilitated by the observation by Loveless[26] that it was thermostable and remained active when the skin-sensitizing antibody was destroyed by heat. When blocking antibody is thus separated from skin-sensitizing antibody, or the skin-sensitizing antibody is inactivated by heat, its presence may be readily demonstrated by a number of

methods. For example, if the heated serum thought to contain blocking antibody is mixed with unheated serum and ragweed in the neutralization procedure, the neutralization point is changed by the inactivation of a certain amount of the antigen by the blocking antibody.

In the middle of Table VI blocking antibody has been added to the system; above is a similar test with the serum from the same patient before treatment; and below is a test with a normal serum. It is apparent that the neutralization point is altered by the addition of the blocking antibody. Equally important is the fact that the serum taken before treatment does not contain any blocking antibody; the blocking antibody appears to be a different antibody, produced as a result of the treatment, and not an altered form of the sensitizing antibody produced by the heating of the serum.

This blocking activity can be demonstrated more directly by simply mixing the blocking serum with the antigen and testing the mixture in various dilutions on skin-sensitized skin sites. Table VII shows the effect of testing a series of uniform, passively sensitized skin sites with a mixture of antigen and serum taken before treatment with ragweed; serum from the same patient after treatment with ragweed; and normal serum, all of these sera being heated for four hours at 56 degrees and then mixed with the ragweed antigen. It is apparent that the blocking antibody inhibits the reaction of the sensitizing antibody and the antigen, and also that the serum of the patient taken before treatment does not contain blocking antibody.

TABLE VII. INACTIVATION OF RAGWEED POLLEN EXTRACT BY HEATED SERUM OF TREATED HAY FEVER PATIENT

| UNITS | AHE. A SERUM HEATED + LR | AHE. P SERUM HEATED + LR | NORMAL SERUM HEATED + LR |
|---|---|---|---|
| 1 | ++ | 0 | ++ |
| 1-3 | + | 0 | + |
| 1-9 | + | 0 | + |
| 1-27 | + | 0 | + |
| 1-81 | ± | 0 | ± |
| 1-243 | 0 | 0 | 0 |

(All sites prepared with Hyl. serum A (1-10), tested after two days with mixtures of heated serum and ragweed extract.)
A serum = before treatment.
P serum = after treatment.
(After Sherman: *J. Immunol.* **40**: 289, 1941.)

The blocking antibody also does not form a precipitate with antigen. It does not produce passive anaphylaxis in guinea pigs and it does not fix measurable amounts of complement. In other respects, however, it does show a somewhat more orthodox behavior than the skin-sensitizing antibody. It can be readily produced in nonallergic patients by injection of pollen extract, although the doses required may be somewhat higher than for routine treatment of hay fever.

Unlike the skin-sensitizing antibody, but like most other human antibodies, it readily passes through the placenta from the maternal to the fetal circulation.

Menzel and others[27] at Roosevelt Hospital have recently completed a paper showing that, like most antibodies, it migrates with the gamma globulin in the Tiselius electrophoresis.

Its specificity apparently corresponds to that of most other antibodies produced by injections of antigen.

Bukantz and his associates[28] have shown that the blocking antibody of treated hay fever patients inhibits the precipitation of ragweed antigen and rabbit antiragweed precipitating antibody.

Portnoy,[29] in our laboratory, found that it likewise inhibited the fixation of complement by rabbit antiragweed serum.

While both of these reactions are of interest in avoiding the usual passive transfer method, they turn out to be less sensitive than the passive transfer method, so they are not a practical substitute.

The most important question about the blocking antibody is the one that is most difficult to answer. Does it protect the patient against hay fever? We have already noted the blocking antibody appears to bind and inactivate antigen whenever it is mixed with it, and the injection treatment has not been shown to produce any other immunologic change that might be expected to protect the patient against hay fever. I think that there is good evidence that the tolerance of the patient for progressively larger injections of pollen extract may be correlated with the development of blocking antibody, which protects the patient against constitutional reactions.

It is important to remember that the constitutional reaction occurs when the antigen spreads through the blood stream from the site of injection to various shock organs. During this passage through the blood stream, the blocking antibody has ample opportunity to inactivate the antigen. In clinical hay fever, on the other hand, the pollen reaches the sensitized nasal membrane directly, and the question is: does the blocking antibody have any chance to inactivate it?

This is illustrated, to some extent, by another method of demonstrating blocking antibody which was described by Maunsell.[30] She took serum from the patient and mixed it with pollen antigens in various dilutions, made up comparable control mixtures containing the same amount of antigen in saline, and compared these two in tests on the patient's own skin. There were differences in reaction produced by the two mixtures, indicating the presence of blocking antibody in the serum. This illustrated that the blocking antibody was capable of inactivating the antigen if it was mixed with it, but the presence of blocking antibody in the patient's blood stream did not prevent the skin reaction when the antigen-saline mixture was injected. By the same token, one might wonder whether blocking antibody in the blood stream would protect the nasal mucosa in direct contact with pollen.

You all recall many conflicting reports in previous meetings of this Academy as to the relationship of blocking antibody titers to clinical protection. I think it is safe to say that there has been no significant correlation between blocking antibody titers and clinical results. If the blocking antibody is the protective factor—and it may well be—the fact still remains to be proved.

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## DISCUSSION

CHAIRMAN FEINBERG.—On one feature of Dr. Sherman's work, I think that he is correct but misinterpreted. That has to do with the exhaustion phenomenon by dissimilar antigens in a patient who is sensitive to both antigens. We can show very readily, in passive transfer or directly on a patient who is sensitive to antigens, that we can exhaust the reactivity to one antigen and to a nonantigen-releasing substance, such as Compound 48/80.

Furthermore, we can exhaust the reactivity of the skin with a chemical histamine releaser, and we can exhaust the activity to a specific antigen to which the patient is sensitive in another area.

I believe, unless I am told differently so far as details of the experiments are concerned, that these phenomena could be explained on the basis of simple histamine depletion locally; that is, where you inject an antigen with high potency (for which there is high potency antibody), you get a marked reaction, a marked histamine release, so that the weaker antigen no longer reacts, no matter how similar it is. I would like to have you re-examine the experiments with this viewpoint in mind, if you follow me.

DR. SHERMAN.—I realize that this is a very important point in interpreting the results, and that is the reason why I showed the other approach to the method, in which you have a patient sensitive to two antigens, treat him with one, and observe the same change in the skin-sensitizing antibody titer to the one with which he is treated and the one with which he is not treated. That is not open to the same objection.

However, this does not prove that the skin-sensitizing antibody reacts with a multitude of different antigens. It may or it may not. I think it is quite obvious that the skin-sensitizing antibody, from the method of its formation in a patient exposed to many different antigens, may be quite different from antibodies which result from one or several specific stimulations with a pure, or at least a single, antigen.

DR. MARY LOVELESS.—I would just like to bring up the work of Bowman and Walzer with reference to the cross reactions that Dr. Sherman has talked about, because I repeated it and found it to be completely reproducible; in other words, if you have a strong whealing reaction in a skin area, as you would have with the so-called dominant clinical allergen, and then you cross-test this with a weaker one, that site is exhausted and will be definitely hyposensitive, so you get a much smaller wheal. I think that explains it, don't you?

DR. SHERMAN.—Yes, to a large extent.

DR. LOVELESS.—The other thing is the blocking antibody. I think the transfusion experiments which, unfortunately, I did not publish showed quite conclusively that transfusion of large amounts of blocking serum taken from a normal subject who had been immunized to pollen were promptly protective against acute hay fever, and this lasted about the expected time—ten days or so.

The blocking antibody of treated patients does accumulate in the conjunctiva and the nasal membranes, as well as the skin, and, if one does delicate threshold testing, this will definitely heighten the tolerance of all these tissues, and I believe that this is a very logical finding, to fit in with their clinical heightened tolerance.

It is true that the circulating blocking antibody would not have a chance to affect the patient's clinical behavior, but it is the tissue antibody-blocking antibody which does so.

DR. SHERMAN.—I should like to point out that, in my opinion, the type of delicate skin and eye test that Dr. Loveless is describing merely reflects the fact that the patient is not as sensitive to the pollen as he was before his treatment. As for the assumption that this is due to blocking antibody in the tissues, that is merely one good explanation. I think that it would be necessary to get the blocking antibody out of the tissues in order to consider it a definitely proved fact.

DR. LOVELESS.—Well, I mean by transfusion. He did not have it before and this definitely altered the skin and conjunctival susceptibility, which I think is good enough evidence that that is what happened. Also, you find a correlation, crudely, between the amount of increased tolerance in the eye, nose, and skin, along with the deposition of circulating antibody. This could be presumptive evidence that there is some connection.

DR. MATTHEW WALZER.—Has Dr. Sherman, from his experience with reagin, observed the phenomenon that Dr. Siegel demonstrated this morning, in which saline dilution impaired the sensitizing property of the antigen?

DR. SHERMAN.—By our method, we would not observe that, because invariably we have diluted them with saline. Therefore, we would not detect that type of change.

DR. WALTER WINKENWERDER.—In the natural history of hay fever, there are a few patients who have a clinical remission spontaneously without the history of any desensitization. That has always confused me with regard to interpreting what has occurred in terms of the blocking antibody. I wonder whether Dr. Sherman has any explanation for that.

DR. SHERMAN.—It is hard enough to say whether or not the blocking antibody is the factor when the change occurs as the result of treatment. Obviously, it is not the factor when it occurs without treatment. There are probably dozens of factors that affect the patient's reaction to pollen, and the blocking antibody is one which may. We have not yet admitted that it is conclusively proved that it does, but there are, obviously, others that definitely do.

# Original Articles

## FORMALINIZED POLLEN TANNATE IN DESENSITIZATION TREATMENT

HYMAN L. NATERMAN, M.D., BROOKLINE, MASS.

### INTRODUCTION

VARIOUS methods to increase the ease, safety, and effectiveness of parenteral pollen desensitization have been used, such as (1) emulsification of pollen extract solutions in oil,[1] (2) addition of gelatin to pollen extract solutions,[2] (3) tannic acid or hydrochloric acid precipitates of pollen extract suspended in water,[3] and (4) tannic acid precipitates suspended in oil with adjuvants.[4] These methods depend upon the menstruum or upon changes in the physical state of the antigen to slow absorption.

Acid precipitates of pollen protein prepared by these methods have relatively little or no immunologic denaturation, as is shown by their ability to produce potentially dangerous, prompt, local and general reactions. A method of denaturing pollen precipitates which would reduce or eliminate immediate reactivity without destroying antigenicity would make it possible to give larger doses. Formalin, which has long been used to denature antigens without destroying antigenic functions, seemed worth trying. In 1948, fourteen ragweed-sensitive patients treated with formalinized ragweed tannate showed small local reactions with large doses. In succeeding years, partially formalinized pollen tannates were used with good desensitization. It was decided to treat a large series of patients in 1954 and 1955 with fully formalinized pollen tannates; the present article describes the methods of preparation, use, and clinical results in 355 pollen seasons, in 193 patients.

### METHODS

*Preparation of Formalinized Pollen Tannate (Pollen-FT).*—Short ragweed pollen was used in making the ragweed extract; equal parts of timothy and orchard grass were used for the grass extract. Birch pollen and oak pollen were processed separately and the formalinized tannates were combined to make the tree antigen.

A 1:20 phenolized saline pollen extract was made in the usual manner and passed through filter paper. Zinc chloride was added to a concentration of

From the Allergy Clinic, Beth Israel Hospital, Boston, Massachusetts; the Department of Medicine, Harvard Medical School; and the Department of Medicine, Tufts Medical School.

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0.1 per cent. Tannic acid (reagent grade in freshly prepared solution) was added to make a concentration of 0.5 per cent or 1.0 per cent. Flocculation of pollen tannate occurred promptly, and the sediment was collected by centrifugation.

In 1954, the pollen tannate was resuspended in saline (sediment from 50 grams of pollen in 200 ml. of saline) and U.S.P. formalin was added to make a 2 per cent concentration. After standing at room temperature for six days, the supernatant was removed and the sediment was lyophilized. The resulting powder was passed through a 200-guage screen, and its nitrogen content was determined by the micro-Kjeldahl method. The powder, as needed, was suspended in phenolized saline with 1 per cent aluminum hydroxide, prepared in the following manner: 4 per cent aluminum chloride in saline was neutralized with sodium hydroxide and autoclaved; phenol to 1 per cent was added. Dilutions of this stock 4 per cent aluminum hydroxide were made as needed. For clinical use, enough pollen-FT was added to 1 per cent aluminum hydroxide to make 1.0 mg. of nitrogen per milliliter, tested for sterility, and called Concentration A. Tenfold dilutions in phenolized saline were made so that B = 0.1 mg. N/ml., C = 0.01 mg. N/ml., and D = 0.001 mg. N/ml.

For 1955, after collecting the tannic acid precipitate as above, the nitrogen content was determined, and the volume was adjusted so that 1 ml. = 1.0 mg. N. Formalin was added to make a 0.5 per cent concentration, and the preparations were kept at room temperature. After two weeks to six or more months of formalinization, the 1.0 mg. N/ml. concentration was diluted with an equal volume of 0.8 per cent sodium bisulfite solution in saline to neutralize the formalin. An equal volume of a suspension of 0.5 per cent aluminum hydroxide and 0.5 per cent hydrocortisone acetate in saline was then added. The final saline suspension contained:

| | |
|---|---|
| (1) Pollen-FT | 0.25 mg. N/ml. |
| (2) Aluminum hydroxide | 0.25 per cent |
| (3) Hydrocortisone acetate | 0.25 per cent |
| (4) Sodium bisulfite | 0.20 per cent |
| (5) Phenol | 0.88 per cent |

In making the 1954 preparations, a 2 per cent concentration of formalin was selected arbitrarily; pollen tannate treated with this concentration for one day showed moderately decreased skin reactivity on intradermal injection and, after three or more days, marked reduction to complete absence of skin reactivity. A six-day period of formalinization was used. In making the 1955 preparations, it was determined that pollen tannate in formalin concentrations of 0.5 per cent and 0.25 per cent for six days showed marked reduction in skin test reactivity; lesser concentrations were increasingly unreactive. Therefore, a 0.5 per cent concentration of formalin was used. Prolonged contact with this concentration of formalin does not destroy antigenicity; a preparation of ragweed tannate standing in 0.5 per cent formalin at room temperature for six months was capable of producing a systemic allergic reaction.

*Dosage.*—In 1954, the starting dose for previously untreated patients was usually 0.1 ml. of the C dilution. Some patients with large skin reactions were

started with the D dilution. Many patients with previous treatment or with smaller skin reactions were started with the B dilution and some were started with A. The maximum dose was 0.25 ml. to 0.5 ml. of A. The usual schedule of doses (in milliliters) was: (1) C 0.1, (2) B 0.1, (3) A 0.1, (4) 0.25 or 0.5. In 1955, the usual dose schedule (in milliliters) of the 0.25 mg. N/ml. concentration was: (1) 0.1, (2) 0.35, and (3) 1.0. In a few instances, when marked sensitivity was suspected, one-half the usual doses were given. For some patients not prone to general reactions, the 1955 schedule was: (1) 0.2 ml., (2) 1.0 ml. Occasionally a dose was repeated because of a large local reaction.

Injections were given subcutaneously in the upper arms every one to three weeks, with increasing dosage. When the maximum dose was reached, it was repeated once at the usual one- to three-week interval, and thereafter at intervals of one to three months, usually two months, until the season began. The last preseasonal injection usually was within four weeks of the expected onset of the season.

*Selection of Patients.*—Hay fever patients, many with varying degrees of seasonal asthma, were skin tested by the scratch method with pollen extract in 50 per cent glycerin-saline in dilutions of 1:50, 1:500, and 1:5,000. Only patients with moderate to marked skin reactions were selected for treatment. Patients with asthma alone who usually showed slight skin reactions were excluded. Patients able to tolerate large starting doses of pollen extract solutions were also excluded. The tests frequently were repeated during and after treatment. The patients were about equally divided as to sex, and ages were from 4 to 79 years. Each pollen season was considered as a unit; thus, a patient treated for both grass and ragweed pollen was considered statistically as two patient seasons. One hundred and four patients treated in 1954, representing 128 pollen seasons, were also treated in 1955.

## RESULTS

*Number of Injections.*—Thirty ragweed-sensitive patients treated in 1953 with a partially formalinized tannate were given ragweed-FT without a prolonged waiting period. They were classified as perennial, and the injections

TABLE I. AVERAGE NUMBER OF POLLEN-FT INJECTIONS GIVEN TO 118 PATIENTS IN 1954 REPRESENTING 162 PATIENT SEASONS

| | | NUMBER OF PATIENT SEASONS | AVERAGE NUMBER OF INJECTIONS TO MAXIMUM DOSE | AVERAGE TOTAL INJECTIONS |
|---|---|---|---|---|
| Ragweed | Perennial: | 30 | --- | 10.1 |
| | Preseasonal: | | | |
| | No previous treatment | 26 | 4.3 | 9.6 |
| | Previous treatment | 36 | 4.05 | 8.1 |
| | Total | 62 | 4.15 | 8.8 |
| Grass | No previous treatment | 15 | 4.4 | 6.6 |
| | Previous treatment | 29 | 4.4 | 6.7 |
| | Total | 44 | 4.4 | 6.7 |
| Trees | No previous treatment | 12 | 3.9 | 5.1 |
| | Previous treatment | 14 | 4.2 | 6.0 |
| | Total | 26 | 4.06 | 5.6 |

given from October, 1953, to October, 1954, were considered the season's treatment; the average number of injections was 10.1 (Table I). From October, 1954, to October, 1955, sixty-six patients were given perennial ragweed treatment; the average number of injections was 6.5 (Table II). Seven grass-sensitive and six tree-sensitive patients had perennial treatment in 1954 and 1955; the average number of injections was 5.4 and 6.0, respectively (Table II).

TABLE II. AVERAGE NUMBER OF POLLEN-FT INJECTIONS GIVEN TO 162 PATIENTS IN 1955 REPRESENTING 193 PATIENT SEASONS

| | | NUMBER OF PATIENT SEASONS | AVERAGE NUMBER OF INJECTIONS TO MAXIMUM DOSE | AVERAGE TOTAL INJECTIONS |
|---|---|---|---|---|
| Perennial | Ragweed | 66 | -- | 6.5 |
| | Grass | 7 | -- | 5.4 |
| | Tree | 6 | -- | 6.0 |
| Preseasonal | Ragweed | 65 | 3.0 | 6.1 |
| | Grass | 36 | 3.0 | 5.9 |
| | Tree | 13 | 2.8 | 5.7 |

In considering the 1954 preseasonal patients, those with no desensitization the previous year, or who never had desensitization treatment, are presented separately to eliminate the possible persistence of desensitization from the previous treatment (Table I). No significant difference is noted when compared with those patients who did have previous treatment. The average number of injections to reach the maximum dose was ragweed, 4.15; grass, 4.4; and trees, 4.06. The average total number of injections was ragweed, 8.8; grass, 6.7; and trees, 5.6. In 1955 (Table II), because it was found safe to start with bigger doses and because of the experience gained in 1954, the number of injections was reduced. The average required to reach the maximum dose was ragweed, 3.0; grass, 3.0; and trees, 2.8. The average total number was ragweed, 6.1; grass, 5.9; and trees, 5.7.

*Local Reactions.*—The injections of pollen-FT were as painless as pollen solutions. The local reactions developed slowly and usually reached a maximum twenty-four to forty-eight hours after injection, which was slight to moderate in degree. The edematous swelling usually cleared in two to four days, leaving a central firm area which gradually shrank to a pea-sized nodule in one week. If there was a marked local reaction, the central nodule would be larger and persist for four to eight weeks, and at times a bluish discoloration of the skin would develop, suggesting a deep-seated ecchymosis. No necrosis or other complications have developed. A dose which gave a large reaction, usually the maximum dose, when repeated invariably caused a much smaller reaction. In 1955, hydrocortisone acetate was added to reduce the induration resulting from intense specific local reactions, with great success.

*General Reactions.*—Some patients who were highly reactive and prone to general reactions had mild general reactions with the maximum dose, consisting of a few wheals which might be scattered or localized to the opposite arm. Occasionally mild nasal congestion also occurred. Such reactions often did not recur with repetition of the maximum dose. No reduction in dose was made

since the reactions were so mild. They occurred from thirty minutes to several hours after injection and subsided spontaneously in from one to several hours, or were relieved promptly by an antihistamine tablet. In 1954, when increases in dosage were five- or tenfold, three female patients had moderate to severe generalized urticaria several hours after injection. No patient had the acute respiratory difficulty characteristic of severe general reactions following injection of pollen extract solution.

TABLE III. CLINICAL RESULTS IN 350 OF 355 PATIENT SEASONS TREATED IN 1954* AND 1955† WITH POLLEN-FT

| | | NUMBER OF PATIENT SEASONS | SATISFACTORY | | UNSATISFACTORY | |
|---|---|---|---|---|---|---|
| | | | NUMBER | PER CENT | NUMBER | PER CENT |
| 1954 | Ragweed | 92 | 88 | 96 | 4 | 4 |
| | Grass | 44 | 41 | 93 | 3 | 7 |
| | Trees | 26 | 24 | 93 | 2 | 7 |
| 1955 | Ragweed | 126 | 107 | 85 | 19 | 15 |
| | Grass | 43 | 43 | 98 | 1 | 2 |
| | Trees | 19 | 18 | 95 | 1 | 5 |
| Total | | 350 | 320 | 91 | 30 | 9 |

*Representing 118 individual patients
†Representing 161 individual patients.

*Symptomatic Relief.*—After careful questioning regarding the nature, frequency, and severity of symptoms and the amount of medication taken after treatment as compared with previous years, the patients were divided into satisfactory and unsatisfactory groups (Table III). The satisfactory group had relief of symptoms ranging from complete with no medication needed to moderate with small doses of antihistamines needed at times. Patients who had little or no change, as compared with previous years, and needed full doses of antihistamines regularly were placed in the unsatisfactory group. In 1954, the results known in 162 patients show satisfactory results in 96 per cent of ninety-two ragweed-sensitive patients, 93 per cent of forty-four grass-sensitive patients, and 93 per cent of twenty-six tree-sensitive patients. In 1955, satisfactory results were obtained in 85 per cent of 126 ragweed-sensitive patients, 98 per cent of forty-three grass-sensitive patients, and 95 per cent of nineteen tree patients. The results in five patients were not obtained. Among 350 patient seasons, therefore, the results were satisfactory in 91 per cent.

## DISCUSSION

The usual pollen desensitization therapy depends on the subcutaneous injection of specific antigen in increasing quantities so that the patient eventually tolerates the injection of a larger amount of antigen with the same or less local reaction than when treatment was begun. The rate of increasing dosage is restricted by the need to avoid distressing local and dangerous general reactions. The methods currently used to slow absorption and thus avoid these undesirable reactions are not entirely satisfactory. They depend on physical or chemical measures which do not alter the reactivity of the antigen. Excessive reactions, therefore, may occur due to unpredictable changes in the physical

state of the preparation on standing or variation in the rate of absorption after injection. For example, radioactive protein in Freund's adjuvant had an increased absorption rate in the first twenty-four hours averaging up to 30 per cent of the total injected and a much slower rate thereafter.[5] The use of an antigen whose capacity for immediate reaction has been reduced or eliminated is safer. An antihistamine in the preparation would increase this safety.

Formalin has long been used to make vaccines and toxoids without loss of antigenicity. Its principal action is thought to be the production of methylene bridges between free protein amine groups, binding protein molecules together. When pollen tannate was formalinized for three days or more, and intradermal skin tests were done, the immediate reactions were absent or greatly reduced. The same material injected subcutaneously did produce reactions after a period of several hours, suggesting that the specific antigen links were blocked by formalinization and gradually released by enzymatic action. Because of this gradual release, the possibility of an overwhelming acute anaphylactic reaction is greatly reduced. That formalinized pollen tannates do desensitize is indicated by the following observations: (1) A large local reaction becomes much smaller when the dose is repeated; (2) frequently the local reaction to an increased dose is less than to the previous smaller dose; (3) frequently the scratch skin test with pollen solutions is markedly reduced after treatment; and (4) satisfactory clinical relief is obtained.

In 1954 pollen-FT was stored in the lyophilized state; in 1955 pollen-FT was stored as 1 mg. N per milliliter in 0.5 per cent formalin at room temperature. No apparent difference could be detected in clinical use. A preparation stored in 0.5 per cent formalin for more than six months, when processed for clinical use, still showed activity. By storing lyophilized pollen-FT, the deterioration of activity in solutions may be avoided with less danger of reactions when different preparations are to be used. It is likely that the same advantage will be evident with the preparations stored in 0.5 per cent formalin.

When pollen-FT is suspended in saline, some of the particles are small enough to form a colloidal suspension. It is likely that the small colloidal particles disperse more readily and react more quickly than larger aggregates. Aluminum hydroxide was used to counteract this and to produce a flocculent type of precipitate.

In 1954, with larger doses of lyophilized FT, intense local reactions would occur at times with resulting large nodules absorbing slowly. Such reactions were specific and not of the "foreign body" type since, when the same dose was repeated, the reaction was invariably much less. In 1955, the addition of hydrocortisone in 0.25 per cent concentration practically eliminated local nodule formation. The maximum quantity of hydrocortisone injected was 2.5 mg., a quantity too small to exert any significant general effect.

The experience of 1954 indicated that a starting dose of 0.01 mg. N and a maximum dose of 0.25 mg. N, would give satisfactory clinical results. This spread of dosage could be obtained with the one concentration of 0.25 mg. N per milliliter used in 1955. The usual starting dose was 0.1 ml. (0.025 mg. N);

in rare instances, when previous susceptibility to general reactions was known or suspected, the starting dose was 0.05 ml. (0.012 mg. N). Later in the year, patients who had had treatment previously and who were not susceptible to general reactions were started with 0.2 ml. (0.05 mg. N); one patient, by error, was given initially 1.0 ml. with no excessive reaction. With threefold to fivefold increases in dosage, the maximum dose of 1.0 ml (occasionally 0.5 ml.) was reached with the second or third injection. The total number of injections given was about six for either the preseasonal or perennial forms of treatment; there is no indication that one type of treatment was better than the other. Many patients who started treatment late did not have the maximum dose repeated as often, and obtained satisfactory results with three or four injections.

Hitherto, only patients with moderate to severe sensitivity have been selected for treatment with pollen-FT. This includes the majority of pollen-sensitive patients. The smaller group of patients with slight sensitivity, who presumably require large doses for effective desensitization, may not get enough antigenic stimulus from the slowly absorbing pollen-FT. However, this group of patients can be treated easily with regular pollen extract solutions. They reach a high level of dosage, up to 0.5 c.c. of a 1:20 pollen dilution, with comparatively few injections and slight risk of general reactions. Probably nothing would be gained by treating such patients with pollen-FT unless much larger doses than those used here were tried.

The possibility of using pollen-FT in various media, such as oil with adjuvants, or gelatin, to delay absorption further has been considered and has been tried to some extent. The difficulties involved in making and injecting such combinations seem to outweigh any advantages. Furthermore, it is uncertain that they would delay significantly the absorption of pollen-FT. For example, radio-iodinated serum albumin processed like pollen-FT and suspended in various media showed about the same rate of absorption from saline suspensions as from oil or gelatin suspensions.[6] Implanting a pellet of pollen-FT might slow absorption enough to permit a one-dose treatment.

The application of this formalinized tannate technique to other preparations is possible. House dust and other allergens may be found suitable. Protein hormones, such as pitressin, insulin, and adrenocorticotrophin, if their specific activity is not destroyed, might have a prolonged action. The danger of inducing or increasing sensitivity when animal proteins (for example insect antigen) are used in this way must be considered carefully. There is no indication that increased sensitivity to the pollens used has occurred.

Four main advantages of pollen-FT over the solutions generally used in pollen desensitization are: (1) one concentration may be used, rather than three or more; (2) a more flexible time schedule of injections can be followed; (3) the danger of general reactions is markedly reduced; and (4) the number of injections is decreased to one-third or less. The clinical results with pollen-FT compare favorably with the results obtained by any other method of pollen

desensitization. Controlled studies on a large scale would be required to determine the relative merits. The large number of patients successfully treated in this study over a two-year period suggests that the satisfactory clinical results were not coincidental and that a controlled study is warranted.

## SUMMARY AND CONCLUSIONS

1. Pollen antigens precipitated from aqueous solutions by tannic acid and zinc and then formalinized (pollen-FT) showed reduced immediate reactivity and prolonged absorption, permitting the injection of large doses.

2. Pollen-FT was used in 1954 and 1955 in the treatment of 193 patients representing 355 tree, grass, or ragweed pollen seasons. The usual maximum dose of 0.25 mg. N (25,000 protein N units) could be reached in three injections. The total number of injections per season was reduced to about six. Satisfactory clinical results were obtained in 91.4 per cent of 350 patient seasons.

3. The advantages of pollen-FT suspensions over other methods of slowing absorption are discussed. The advantages over the pollen extract solutions commonly used are: (1) the use of a single concentration; (2) a more flexible time schedule of injection; (3) reduced danger of general reactions; and (4) reduction of number of injections to one-third or less.

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# ALLERGIC SENSITIZATION OF THE SKIN AND NAILS TO ACRYLIC PLASTIC NAILS

ALEXANDER A. FISHER, M.D., ANDREW FRANKS, M.D., AND HAROLD GLICK, M.D.,
NEW YORK, N. Y.

IN RECENT months there has been made available plastic material which can be fashioned into artificial "finger-nails" that are advertised "for those who want long nails." In addition, the plastic nails may be used by persons who wish to disguise dystrophic or diseased nails or who wish to discourage nail biting. This plastic material is essentially the same as that used in acrylic dentures, and is marketed as a liquid monomer and powder polymer. The liquid monomer is methyl methacrylate, and the powder is polymethyl methacrylate. One manufacturer claims that the material is completely harmless. However, other manufacturers warn that allergic reactions to this material may be encountered.

The acrylic liquid and powder used in artificial "nails" do not require heat for polymerization, but will polymerize and harden at room temperature. These self-curing acrylic resins are created by inducing polymerization of the mixture of methyl methacrylate monomer and polymethyl methacrylate powder with an organic peroxide and an accelerator or promoter. The self-cured resins are not quite as hard as those resins polymerized at high temperature, and they normally contain a somewhat higher residual monomer content than do the heat-cured resins. Nevertheless, these acrylic resins can be fashioned to form excellent plastic nails which remain intact for several weeks.

In a previous communication,[1] one of us (A. A. F.) pointed out that methyl methacrylate liquid monomer is a potent sensitizer and can cause allergic contact type of eczematous reactions on the skin and the oral mucosa. When the plastic acrylic "nails" became available, we predicted that allergic reactions to this material would occur. In a short time we were able to observe four cases of allergic eczematous contact reactions of the skin, onychial, and paronychial tissues due to acrylic plastic "nails." Some of the reactions were rather severe and painful and caused nail changes which lasted for several months. In each case, we were able to prove that it was the liquid monomer that was the sensitizing agent. The powder polymer was inert, allergenically speaking.

## CASE REPORTS

CASE 1.—One of the authors (H. G.) a 34-year-old man, had applied acrylic plastic "nails" to four of his fingers to cover a long-standing onychodystrophy due to *Trichophyton rubrum*. These plastic "nails" were reapplied at two-week intervals. After the fourth

From the Department of Dermatology and Syphilology of the New York University Post Graduate Medical School and the Skin and Cancer Unit of the University Hospital.

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application a severe itching sensation was noted in the interdigital webs. This was soon followed by a marked edema of the paronychial tissue and severe subungual throbbing and pain.

Immersion of the fingers in ice water relieved the swelling and pain somewhat, but it was impossible to remove the plastic nail mixture. Vesiculation of the sides of the fingers was observed the following day. One per cent hydrocortisone ointment relieved the severe itching of the fingers and the webs. The severe onychial and paronychial pain persisted for forty-eight hours and necessitated the use of analgesics.

Patch tests with the liquid monomer revealed a very strong reaction within two hours, consisting of marked edema and vesiculation. Patch testing with the polymer showed no reaction. Patch testing of the monomer on four controls showed no reaction.

The paronychial swelling subsided in about ten days. The affected nails remained tender and brittle for two weeks and then returned to their original onychodystrophy.

Two months later, when this sensitized physician attempted to patch test one of his patients with the acrylic monomer, he immediately felt an itching sensation in the finger webs and developed a mild contact dermatitis of the finger, despite the fact that he took precautions not to come in actual contact with the acrylic monomer. The monomer is somewhat volatile, so that actual contact with the liquid substance itself is apparently not necessary to cause reaction in highly sensitive persons. The vapor emanating from an open bottle of acrylic monomer was capable of causing dermatitis in this sensitized person.

Fig. 1.—Residual dystrophy three months after first and only application of acrylic nail plastic. Both thumbnails (not shown), all the nails of the right hand and the left third and fifth fingernails remained dystrophic. The left second fingernail did not have any plastic applied. The left fourth fingernail shows approximate condition of all the nails before the acrylic plastic had been applied.

CASE 2.—A 40-year-old woman applied acrylic plastic to nine of her nails because the nails were short, and broken off at the distal ends. Forty-eight hours later, marked swelling and pain of the paronychial and subungual tissues occurred. A dermatitis appeared on the dorsa of the affected fingers. Fig. 1 shows the condition of the nails three months after the acrylic material had been applied. The left index finger had always been normal and no plastic had been applied. The left fourth finger, which apparently had been least affected, shows the best recovery and portrays the approximate condition of the nails before

the application of the acrylic plastic. The other nails, including those of the thumbs, show a marked dystrophy and moth-eaten appearance of the nail plate. Almost complete lysis of the affected nail plate had occurred. The dorsal aspect of some of the fingers shows a healing scaling dermatitis. Scraping of the nails revealed no evidence of fungi. The patch test with acrylic monomer was strongly positive. The polymer produced no reaction.

CASE 3.—A woman, aged 30 years, was employed as a demonstrator for the application of acrylic plastic "nails." Her own nails were normal. For four months she applied the material to her own nails and instructed others on how to make the applications. At the end of this four-month period she suddenly developed redness and itching about the nails, and a severe paronychia developed. A vesicular dermatitis of the right cheek and eyelids also became evident. The distal portion of the nails separated slightly from the nail bed. One month later the nails had returned to normal. Patch test with the acrylic monomer was strongly positive.

CASE 4.—A 26-year-old woman was also employed as a demonstrator for the application of acrylic plastic "nails." Her own nails had always been considered normal. She would demonstrate the use of the acrylic "nails" by applying the plastic to her own nails. At the end of the two-month period she developed itching and a vesicular dermatitis of the tips of the fingers. The following day a moderately severe paronychia developed on those fingers that she used for demonstration purposes. She avoided further contact with the acrylic plastic material. The finger dermatitis and the paronychia subsided within three weeks. Patch testing with the acrylic monomer showed a strongly positive reaction. The polymer produced no reaction.

## COMMENT

In this series of cases, all four patients reacted strongly to the acrylic liquid monomer on patch testing. We[1] had previously shown that this substance is not a primary irritant and that a positive patch test usually means clinical hypersensitivity. None of the patients studied were hypersensitive to the polymer powder. This agrees with our previous findings with regard to the acrylic polymer used in dentures.

It should be noted that in Cases 3 and 4 the patients had normal nails originally. Both had been employed as demonstrators of this method of applying "artificial nails." One became sensitized in two months, the other in four months. The nail changes in these two patients were not severe, and restitution to normal took place in three weeks. The first patient was a physician with onychomycosis who had a very painful reaction and had become sensitized within a two-month period. The onychomycosis itself was not affected by the allergic reaction. It should be noted that the second patient showed symptoms within forty-eight hours after the first application of the acrylic plastic. Apparently she somehow had previously become sensitized to this material. Severe nail changes were still present three months after the initial application of the acrylic material. None of the paronychial reactions became pustular or required incisions.

"Pre-use" patch testing, except possibly in the second case, would not have revealed allergic sensitivity since the sensitivity developed only after the use of the material for from two to four months. Spealman and associates[2] state that the monomer methyl methacrylate has been shown to have a sensitizing index of 30 per cent. We have not attempted to confirm this statement, but we agree that the monomer is a potent sensitizer and frequently may cause clinical

symptoms. We also found that the completely polymerized acrylic material, such as heat-cured acrylic dentures and Lucite or Plexiglass, is apparently inert.[1, 3] MacKay,[4] who used acrylic implants for repairing cranial defects, considers acrylic plastic substances to be "biologically entirely inert and innocuous." We believe that this statement is correct, provided that the acrylic plastic is cured by heat and complete polymerization takes place so that no residual monomer remains. If a method could be devised for curing acrylic "nails" by heat, we believe that such nails would not sensitize or cause clinical symptoms.

It is interesting, at this point, to compare the clinical picture caused by acrylic plastic material used on the nails with that caused by "undercoat" plastic. In 1948 and 1949 several reports of nail changes were published showing the effects of the application of a plastic consisting of phenol formaldehyde and synthetic rubber to the nails. This combination of plastics, which was applied to allow better adherence of nail polish and to prevent flaking and chipping, was called a plastic nail "undercoat."

Fig. 2 compares in table form the salient feature of reactions due to acrylic plastic applied to the nails with those due to the phenol formaldehyde-synthetic rubber nail "undercoat."

| REACTION | ACRYLIC PLASTIC "NAILS" | PHENOL FORMALDEHYDE AND SYNTHETIC RUBBER NAIL "UNDERCOAT" |
|---|---|---|
| Paronychia | Always present and usually severe | Infrequent |
| Dermatitis | Fingers, face, and eyelid dermatitis may be present | Not reported |
| Subungual hemorrhage and nail discoloration | None | Frequently present |
| Residual nail dystrophy | Disfigurement of nail may continue for several months | Restitution to normal usually in two months, but the changes may continue much longer |
| Subjective symptoms | All cases had severe onychia and paronychial pain | Most cases symptomless, but some with very severe and painful onychia and paronychia |

Fig. 2.—Comparison between reactions to acrylic plastic "nails" and plastic nail "undercoat."

It will be noted from Fig. 2 that the reactions from the acrylic are much more frequently drastic and tend to be of longer duration than those reported from the nail "undercoat." There were no permanent nail changes from the nail "undercoat." Further observation will be necessary to determine whether the majority of the nail changes due to acrylic plastics will be of very long standing or even of a permanent nature.

Another type of disturbance of the nail and nail bed was described by Frumess, Lewis and Henschel.[7] They described one case with symptoms similar to those caused by the "undercoat." The cause of the disturbance in their case was the application of artificial fingernails which contained nitrocellulose and was applied with an adhesive substance containing cellulose nitrate

dissolved in butyl acetate and an unidentified plasticizer. These authors felt that there was a close relationship between the nail disorder due to "base coat" and that described in their report.

## SUMMARY AND CONCLUSIONS

Four cases of onychia, paronychia, and dermatitis are reported which followed the application of acrylic plastic material to the nails. The plastic consists of a liquid monomer and a powder polymer. Patch tests revealed that all four cases showed a marked allergic reaction to the acrylic liquid monomer (methyl methacrylate). None reacted to the powder polymer (polymethyl methacrylate). The acrylic monomer is essentially the same as that used in dentistry and is a potent sensitizer. When sensitization occurs, severe onychia and paronychia occur and dystrophic nail changes may persist for several months. Diagnostic features of the reactions due to acrylic monomer are compared with those due to phenol formaldehyde rubber resin nail "undercoat."

Some patients followed for three months after the application of the acrylic plastic to the nails still showed dystrophic nail changes. Further observation will be necessary to determine whether these changes are temporary or permanent.

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# ALLERGY TO CHYMOTRYPSIN

WALTER R. MACLAREN, M.D., AND FREDERICK ALADJEM, PH.D.,* PASADENA, CALIF.

THIS report presents a case of allergic sensitivity to chymotrypsin (bovine) in a laboratory worker. The sensitivity apparently was induced by inhalation of the powdered material.

With the increasing use of purified enzyme preparations, the hazard of sensitization should be recognized. A larger number of scientific workers are being exposed to inhalation of the dried materials. Injectible chymotrypsin is being used to reduce tissue swelling following trauma and infection.[1] The other pancreatic proteolytic enzyme, trypsin, is used medically, both by injection in inflammatory disorders[2, 3] and in the form of an aerosol to liquefy mucus in cases of asthma, bronchitis, and bronchiectasis.[4, 5]

Chymotrypsin is produced by the action of trypsin on chymotrypsinogen. It acts on the peptide link in the carboxylic side of aromatic amino acids. Because of the proteolytic action, the enzymes have been used to dissolve mucus or fibrin.

During tests for specific skin sensitivity, the proteolytic activity of trypsin and chymotrypsin, with its resulting irritative wheal and flare, introduces the factor of nonspecific reactions, and this must be evaluated in each individual test. To reduce this source of error, we used the nonproteolytic precursor of chymotrypsin (chymotrypsinogen) in the passive transfer tests reported below.

Chymotrypsinogen and chymotrypsin have been reported to be distinguishable by means of the Schultz-Dale reaction in the guinea pig;[6] however, they are very closely related immunologically. Their cross reactivity is sufficiently strong to have permitted the use of chymotrypsinogen to test for chymotrypsin sensitivity in the case reported here.

## CASE HISTORY

A 28-year-old white male graduate student noted sneezing and itching eyes in the fall of 1955 whenever he was engaged in weighing out chymotrypsin powder. He had been working regularly with the material, mainly in solution, for about three years. A scratch test with chymotrypsin in 1/100 dilution (1.0 per cent) was said to have been positive one day but negative when the test was repeated the following day. He was given a dust mask to wear while weighing the powdered material.

The patient did not know of any close relatives with symptoms of allergic disease. As a child he had suffered frequent colds, and in late childhood and the early teens had severe nasal allergy from exposure to hay and feed on a farm in northern Washington.

From the Department of Medicine (Allergy), University of Southern California School of Medicine, and the Gates and Crellin Laboratories of Chemistry, California Institute of Technology.

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*Postdoctoral Fellow, National Microbiological Institute, United States Public Health Service.

After coming to Southern California, the patient became sensitive to olive pollen, exposure to which resulted in swollen eyes, nasal congestion, and sneezing and coughing at night. Intracutaneous tests gave positive reactions to several grass and weed pollens, and some antigen injections were tried with moderate success. He was not sensitive to beef protein, either clinically or by skin test.

In December, 1955, he was tested intracutaneously with 0.25 per cent chymotrypsin solution. Approximately 0.01 ml. of solution or 0.025 mg. of the enzyme was injected. Within fifteen minutes he complained of itching of the nose and throat, and showed a 4 plus local reaction. He was seen fifteen minutes later with swollen eyes and uncontrollable sneezing; he was dyspneic, slightly cyanotic, sweating, and feeling weak and faint.

He was judged to be having a "constitutional reaction" to the injected chymotrypsin and was given slowly 10 mg. Benadryl by vein and 0.2 ml. of epinephrine 1/1,000 subcutaneously. The epinephrine was repeated in a few minutes and his condition began to improve gradually. After resting for an hour, he was back to normal, except for a sense of fatigue.

*Skin Testing.*—Serum was drawn from the patient four hours after the reaction and again seven days later. These sera were stored in the frozen state and tested three weeks later by passive transfer for the presence of skin-sensitizing antibody.

Skin sites on three nonallergic young adults were injected with 0.1 ml. of both the four-hour and seven-day sera. Twenty-four hours later these sites were challenged with 0.05 per cent chymotrypsinogen of bovine origin.

Two donors reacted with 3 plus wheal and erythema at the sites of the four-hour serum, and with 4 plus wheal and erythema at the seven-day-serum sites. Controls were negative in these two subjects. In the third subject both sera and the antigen control sites gave equal but slight reactions.

The patient was not tested directly with chymotrypsinogen. No precipitating antibody against chymotrypsinogen was detectable in either serum. Neither could passive anaphylaxis to chymotrypsinogen in the guinea pig be demonstrated, using either serum.

Our results show that exposure to bovine chymotrypsin can produce strong allergic sensitization, with transferable skin-sensitizing antibodies against the enzyme. It is logical to suppose that related enzymes, such as trypsin, can have the same effect in susceptible persons. We suggest that when proteolytic enzymes are to be used in treatment, particularly by injection, a sensitivity test be performed first. This would be an important precaution to take if the enzyme had been used before in the same patient. For the reason already discussed, the nonproteolytic enzyme precursor should be used first in skin testing for allergic sensitivity.

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# American Academy of Allergy

## PRECONVENTION SCIENTIFIC ASSEMBLY

**Saturday and Sunday**

**Feb. 2 and 3, 1956**

**The Hotel Statler**
**Los Angeles, California**

UNDER THE AUSPICES OF THE POSTGRADUATE EDUCATION COMMITTEE OF THE AMERICAN ACADEMY OF ALLERGY AND SPONSORED IN PART THROUGH A GRANT BY CIBA PHARMACEUTICAL PRODUCTS, INC.

There will be no charge or registration fee for attending these meetings. To aid in planning future Post-Graduate Reviews, those in attendance will be asked to register.

### I. Panel Discussion: Drug Allergy

**Saturday, Feb. 2, 1957**
9 A.M.-12 M.
**Golden State Room**

*Moderator:*

William B. Sherman, M.D., Associate Professor of Medicine, Columbia University, New York, New York.

*Participants:*

Max Samter, M.D., Associate Professor of Medicine, University of Illinois College of Medicine, Chicago, Illinois.

Robert P. McCombs, M.D., Professor of Graduate Medicine, Tufts University School of Medicine; Senior Physician and Allergist, New England Center Hospital, Boston, Massachusetts.

Merrill W. Chase, Ph.D., Rockefeller Institute for Medical Research, New York, New York.

Edmund L. Keeney, M.D., Medical Director, Scripps Clinic and Research Foundation, La Jolla, California.

The immunologic and clinical phases of drug allergy will be discussed in detail.

### II. Panel Discussion: Bronchial Asthma

**Saturday, Feb. 2, 1957**
1-4 P.M.
**Golden State Room**

*Moderator:*

Oscar Swineford, Jr., M.D., Professor of Medicine, University of Virginia School of Medicine, Charlottesville, Virginia.

*Participants:*

George Piness, M.D., Associate Professor of Medicine, University of Southern California Medical School, Los Angeles, California.

William Paul Thompson, M.D., Clinical Professor of Medicine, College of Medical Evangelists, Los Angeles, California.

Hurley L. Motley, M.D., Professor of Medicine and Director, Cardiorespiratory Laboratory, University of Southern California Medical School, Los Angeles, California.

Ralph Bookman, M.D., Instructor of Medicine, University of Southern California Medical School, Los Angeles, California.

The pathology, pathologic physiology, diagnosis, and differential diagnosis of bronchial asthma will be discussed. The panel will consider the pathologic physiology and treatment of cardial asthma as well as pulmonary function tests, intermittent positive pressure oxygen therapy, emphysema and pulmonary fibrosis, and the differential diagnosis of bronchial asthma in infants and children.

## III. Panel Discussion: Atopic Dermatitis

**Sunday, Feb. 3, 1957**

9-10 A.M.

**Golden State Room**

*Moderator:*

Louis Tuft, M.D., Clinical Professor of Medicine, School of Medicine, Temple University, Philadelphia, Pennsylvania.

*Participants:*

Jerome Glaser, M.D., Assistant Professor of Pediatrics, University of Rochester School of Medicine, Rochester, New York.

Thomas H. Sternberg, M.D., Professor of Dermatology, University of California Medical Center, Los Angeles, California.

H. Elias Diamond, M.D., Chief of the Pediatric Allergy Clinic, Fordham Hospital; Chief, Adult Allergy Clinic, Bronx Hospital, New York, New York.

This panel will discuss all phases of atopic dermatitis, including the immunology and clinical considerations. Local and allergic treatment will be discussed.

## IV. Panel Discussion: Immunology of Allergy

**Sunday, Feb. 3, 1957**

11 A.M.-12 M.

1-4 P.M.

**Golden State Room**

*Moderator:*

Leo H. Criep, M.D., Associate Professor of Medicine, Head of Section on Allergy, Department of Medicine, School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania.

*Participants:*

Linus Pauling, M.D., Gates and Crellin Laboratories of Chemistry, California Institute of Technology, Los Angeles, California.

Frank J. Dixon, Jr., M.D., Professor and Chairman, Department of Pathology, School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania.

Merrill W. Chase, Ph.D., Rockefeller Institute for Medical Research, New York, New York.

Dan Campbell, Ph.D., Professor of Immunochemistry, California Institute of Technology, Pasadena, California.

Hubert Bloch, M.D., Professor and Chairman, Department of Microbiology, School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania.

Sidney Raffel, M.D., Professor and Executive Head, Department of Medical Microbiology, Stanford University School of Medicine, Stanford, California.

The panel will discuss the immunologic basis of allergy, the nature of antigen and antibody, and the immunology of anaphylaxis, atopy, bacterial allergy, and drug allergy.

## SCIENTIFIC PROGRAM

**Monday, Feb. 4, 1957**
**Pacific Ballroom**
**Morning Session, 9 A.M.**

GEORGE PINESS, M.D., CHAIRMAN
A. M. TARGOW, M.D., VICE-CHAIRMAN

1. **Spirometric Studies of Pulmonary Functions: Acetylcholine as a Bronchospastic Agent in Asthma** (Abstract No. 10). D. Edward Frank, M.D., Sun Valley, California.
2. **Studies on Reversed Anaphylaxis (Effect of Cortisone, ACTH, Whole Body Irradiation, and Diet-Induced Complement Deficiency)** (Abstract No. 16). Douglas E. Johnstone, M.D., Rochester, New York.
3. **A Comparative Study of the Efficacy of Intramuscular Epinephrine, Intravenous Prednisolone, and Oral Prednisolone in the Treatment of Bronchial Asthma** (Abstract No. 19). H. H. Pinkerton, M.D., and Thomas E. Van Metre, M.D., Baltimore, Maryland.
4. **Adrenal Function in Allergy. II. Observations of an ACTH-17-hydroxycorticosteroid Response Test Before, During, and After Administration of Steroids in Allergic Children** (Abstract No. 24). Sheldon C. Siegel, M.D., Bailey J. Levin, Jr., M.D., Robert E. Smith, M.D., Robert S. Ely, M.D., and Vincent C. Kelley, M.D., Ph.D., Los Angeles, California.

10 A.M. **Address: "The Dynamics of Adrenal Cortical Activity in Man and Their Relation to Hypersensitivity." Peter H. Forsham, M.D., Associate Professor of Medicine and Pediatrics, Chief of Endocrine Clinic, and Director of Metabolic Unit, University of California School of Medicine, San Francisco, California.**

10:50-11:15 A.M. **Intermission to Register With Exhibitors.**

5. **The Effect of Corticosteroid Administration on Blood Histamine Values** (Abstract No. 18). Joseph W. Noah, M.D., and Alta Brand, St. Louis, Missouri.
6. **Blood Histamine in Thermal Intolerance: Report of a Case of Cryoglobulinemia** (Abstract No. 15). Lowell L. Henderson, M.D., Charles F. Code, M.D., and Grace M. Roth, M.D., Rochester, Minnesota.
7. **Experimental and Clinical Results With Histamine Liberators** (Abstract No. 14). Bernard N. Halpern, M.D., Paris, France.

12-2 P.M. **Luncheon Intermission.**

**Afternoon Session, 2 P.M.**

WILLARD SMALL, M.D., CHAIRMAN
NORMAN SHURE, M.D., VICE-CHAIRMAN

8. **Immune Responses to Staphylococcus Aureus Antigens and Their Etiologic Role in Anaphylactic Reactions and Experimentally Produced Lesions of Vascular Sensitization** (Abstract No. 4). Sheldon G. Cohen, M.D., Daniel S. Dzury, B.S., and Frances J. Michelini, Ph.D., Wilkes-Barre, Pennsylvania.
9. **The Allergist's Stake in Collagen Disease** (Abstract No. 5). Leo H. Criep, M.D., Pittsburgh, Pennsylvania.

10. **Passive Cutaneous Anaphylaxis (P.C.A.) in the Guinea Pig. A Pathologic and Immunologic Study** (Abstract No. 8). J. Prochazka Fisher, M.D., and Robert A. Cooke, M.D., New York, New York.

2:45 P.M. **Address: "Mechanisms of Immunologic Tissue Injury." Russell Weiser, M.D., Professor of Immunology, University of Washington School of Medicine, Seattle, Washington.**

3:30-4 P.M. **Intermission to Register With Exhibitors.**

11. **The Role of the Carrier in the Formation of Complete Antigens** (Abstract No. 17). Rudolf L. Mayer, M.D., Summit, New Jersey.

12. **Enzymatic Mechanisms in the Action of Complement: Possible Relations to Allergy** (Abstract No. 1). Elmer L. Becker, M.D., Chief, Department of Immunochemistry, Division of Immunology, Walter Reed Hospital, Washington, D. C.

**Report of the Committee on Drugs, Research Council of the American Academy of Allergy.** Sidney Friedlaender, M.D., Chairman.

**Tuesday, Feb. 5, 1957**

**Pacific Ballroom**

**Morning Session, 9 A.M.**

WILLIAM P. BUFFUM, M.D., CHAIRMAN
JEROME J. SIEVERS, M.D., VICE-CHAIRMAN

13. **Prophylaxis of Asthmatic Bronchitis in Children With Panmycin—A Controlled Study** (Abstract No. 13). William C. Grater, M.D., Dallas, Texas.

14. **Intrinsic Bronchial Asthma as a Manifestation of Fibrocystic Disease of the Pancreas** (Abstract No. 6). Vincent J. Derbes, M.D., John H. Dent, M.D., and Roy White, Jr., M.D., New Orleans, Louisiana.

15. **Studies in the Antigenicity of Egg Yolk** (Abstract No. 28). Richard H. Todd, M.D., William A. Howard, M.D., Benjamin P. Lafsky, M.D., Clifford A. Webb, M.D., and Stanley I. Wolf, M.D., with technical assistance of Joan Best, Washington, D. C.

16. **Treatment of Hay Fever With Immune Milk** (Abstract No. 23). William B. Sherman, M.D., and Samuel A. Freedman, M.D., New York, New York.

10 A.M. **Address: "The Genetic Basis of Biological Specificity." George W. Beadle, Ph.D., D.Sc., Professor of Biology and Chairman, Division of Biology, California Institute of Technology, Los Angeles, California.**

10:45-11:15 A.M. **Intermission to Register With Exhibitors.**

17. **The Value of Intelligence and Projective Psychological Measurement in the Management of Intractable Asthma of Childhood** (Abstract No. 29). Harold S. Tuft, M.D., and Lawrence C. Johnson, M.A., Denver, Colorado.

18. **The Occurrence of Multiple Disseminated Granulomata in Sensitized Animals** (Abstract No. 3). Merrill W. Chase, Ph.D., Irene S. Slizys, and Raymond A. Allen, M.D., New York, New York.

11:45 A.M.-12:05 P.M. **Address: "The Changing Picture of Allergy." Dr. Ethan Allen Brown, M.R.C.P. (England), L.R.C.P. (London), President, American College of Allergists, Boston, Massachusetts.**

12:15-2 P.M. **President's Luncheon for Members, Wives, and Guests.**

**Afternoon Session, 2:00 P.M.**

ALBERT ROWE, M.D., CHAIRMAN
HENRY T. FRIEDMAN, M.D., VICE-CHAIRMAN

19. **Respiratory Allergic Disease in an Industrial Population** (Abstract No. 11). William Franklin, M.D., and Francis C. Lowell, M.D., Boston, Massachusetts.

20. **Primary Pulmonary Emphysema in Middle-Aged Adults** (Abstract No. 22). Paul M. Seebohm, M.D., George W. Bedell, M.D., and June M. Fisher, M.D., Iowa City, Iowa.

21. **Cardiac Asthma—Some Practical Considerations** (Abstract No. 26). Oscar Swineford, Jr., M.D., H. Rowland Pearsall, M.D., and Lucius G. Gage, M.D., Charlottesville, Virginia.

2:45 P.M. **Address: "Biophysical Characterization of Reaginic and Blocking Sera." John R. Cann, M.D., Associate Professor of Biophysics, University of Colorado Medical Center, Denver, Colorado.**

3:30-4:00 P.M. **Intermission to Register With Exhibitors.**

4:00 P.M. **Annual Business Meeting of the American Academy of Allergy, Pacific Ballroom.**

**Wednesday, Feb. 6, 1957**

**Pacific Ballroom**

**Morning Session, 9 A.M.**

WILLIAM B. SHERMAN, M.D., CHAIRMAN
RALPH BOOKMAN, M.D., VICE-CHAIRMAN

22. **In Vitro Demonstration of an Antibody-Like Factor in Sera of Ragweed-Sensitive Individuals** (Abstract No. 21). Bram Rose, M.D., J. Gordon, M.D., and A. H. Sehon, Ph.D., Montreal, Canada.

23. **Study of the Effect of Pantothenic Acid Upon Allergic Reactions** (Abstract No. 30). Louis Tuft, M.D., Joan Gregory, B.S., M.T., and Donald Gregory, M.D., Philadelphia, Pennsylvania.

24. **Constitutional Reactions From Intradermal Skin Testing** (Abstract No. 31). Paul P. Van Arsdel, Jr., M.D., and William B. Sherman, M.D., New York, New York.

25. **The Enhancement of Infection With Staphylococcus Aureus in Mice Sensitized by Serum-Pertussis Mixtures** (Abstract No. 20). Samuel J. Prigal, M.D., New York, New York.

26. **Arthus Desensitization Studies of the Antibodies, Skin Reactivity and Serum Protein in Lasting Desensitization** (Abstract No. 27). Oscar Swineford, M.D., Cornelia Hoch-Ligeti, M.D., Karen Irvine, B.S., Eliza Fitch, M.D., and Susan Quelch, B.S., Charlottesville, Virginia.

10:15 A.M. **Address: "Identification of Antigens and Cells by Means of Antibodies." Irving Gordon, M.D., Professor and Chairman, Department of Medical Microbiology, University of Southern California School of Medicine, Los Angeles, California.**

11-11:30 A.M. **Intermission to Register With Exhibitors.**

27. **Allergic Treatment in Nasal Polyposis, Its Importance and Value** (Abstract No. 2). George I. Blumstein, M.D. and Louis Tuft, M.D., Philadelphia, Pennsylvania.

28. **A Semi-in Vitro Procedure for Detection of Neutralizing Antibody Induced by Injection Therapy of Ragweed Hay Fever** (Abstract No. 9). Edna M. Follensby, Ph.D., and Francis C. Lowell, M.D., Boston, Massachusetts.

29. **Liquefying Action of Lyzozyme on Asthmatic Sputum and Gastric Mucin and Clinical Trial of Lyzozyme and Trypsin in Asthma** (Abstract No. 7). Bernard Dickstein, M.D., Flint, Michigan.

30. **The Preparation and Standardization of a New Type of Alum-Precipitated Extract of Whole Ragweed Pollen and Its Clinical Evaluation** (Abstract No. 12). Abner M. Fuchs, M.D., and Margaret Strauss, M.S., New York, New York.

31. **Should Patients With Asthma Fly?** (Abstract No. 25). S. Wm. Simon, M.D., Dayton, Ohio.

# Book Review

**Allergy in Childhood.** By Jerome Glaser, M.D. Springfield, Ill., Charles C Thomas, 1956. 529 pages. Price, $12.50.

Most of the books on allergy have attempted to cover the entire field in greater or lesser detail. Only a few have been limited to the problems of allergy in infancy and childhood. This is the largest and most complete treatise on that subject. Since allergy is an important problem in the practice of pediatrics, and since children comprise a large fraction of the patients of most allergists, physicians in both fields should find this book valuable.

For the pediatrician, the book offers a considerable amount of basic material on allergic methods of diagnosis and treatment. However, there is no adequate discussion of the allergic management of asthma and perennial allergic rhinitis or of injection treatment with inhalants other than pollens. Likewise, the reader is referred to other books for even the basic information on the pollens producing hay fever.

The handling of the subject is more suitable for the allergist without special knowledge of pediatrics. The discussions of differential diagnosis are excellent, with special reference to the diseases of infants and children most often mistaken for allergic reactions. The allergic diseases more common in children than adults are also covered in considerable detail.

Despite the omissions mentioned, the book is recommended as the best on its subject.